MONOGRAPHS ON GENERAL, AGRICULTURAL AND INDUSTRIAL MICROBIOLOGY

Edited by

R. E. Buchanan, Ph.D.
E. B. Fred
S. A. Waksman, Ph.D.

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MICROBIOLOGY MONOGRAPHS
GENERAL · AGRICULTURAL · INDUSTRIAL

EDITED BY
R. E. BUCHANAN, *Iowa State College*
E. B. FRED, *University of Wisconsin*
S. A. WAKSMAN, *Rutgers University*

VOLUME ONE

MORPHOLOGIC VARIATION AND THE RATE OF GROWTH OF BACTERIA

BY
ARTHUR T. HENRICI, M.D.
Professor of Bacteriology
The University of Minnesota

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To my father
who first showed me
the marvels revealed
by the microscope.

PREFACE

This book makes no pretense of being a treatise on the morphology of bacteria, but is rather a record of personal researches undertaken with the hope that by the "magic of numbers" some order might be brought out of the chaos which has so far filled that field of bacteriology which has to deal with the form and structure of bacterial cells. I must state frankly at once that I am no mathematician. The expert in biometrics will therefore find here no such carefully fitted curves and elaborate formulæ as delight his heart, but the ordinary person may follow my argument with only a very elementary knowledge of statistics. With so poor a foundation I should have been rather hesitant about publishing this work were I not actuated by a conviction that the use of even such simple statistical methods as I could handle has yielded truth of profound signfiicance not only for bacteriology, but for general biology as well, and by the hope that such publication may stimulate others more thoroughly equipped to enter this field of investigation.

The history of the problem of morphologic variation in bacteria is rather curious. The ideas prevalent in the early days of the science —that bacteria possessed complex life cycles, that the various species were freely transmutable, and that they were all perhaps but stages in the development of some higher fungi—were completely upset by the discovery of methods of pure culture, and replaced by the doctrine of monomorphism. This dogma has dominated the field ever since, being opposed only in recent years by a minority of new pleomorphists. So firmly has the monomorphistic viewpoint been established that research on the problem has been almost completely discouraged; bacteriologists have frequently noted that their organisms appeared different from day to day, but have blindly considered all variations from the textbook picture as pathological, as evidence of injury or death of the cells, or else have considered the variations noted to be of such a haphazard character as to be of no significance. The development of specific biochemical and serological reactions has made possible the identification of organisms

without having recourse to their morphologic characters, and the carrying on of many types of bacteriological investigation without ever using the microscope.

In this work I shall show that, contrary to the orthodox teaching, the cells of bacteria are constantly changing in size and form and structure; but that instead of these changes occurring in a haphazard or meaningless fashion, or instead of being phases in a rather vague and complex life cycle, they occur with great regularity and are governed by relatively simple laws which, after more data have been accumulated and analyzed, may probably be very precisely formulated.

Bacteria have been rather neglected by the general biologists, and in works on general biology one rather gains the impression that bacteria are to an extent organisms apart from the rest of the living world, more or less exceptions to the general biologic laws. This is due in part to the fact that the study of bacteria requires a peculiar technique, but more particularly to the fact that they are so minute in size and simple in form. There is no multiplicity of structures to explain the complexity of function; their very simplicity has repelled rather than invited research. More particularly the absence of a demonstrable nucleus and of sexual reproduction has discouraged investigation. Now this would seem on the face of it illogical; the simplest forms of life should be studied before the complex. Here are the most elemental organisms that can be visually observed, yet we know practically nothing concerning their fundamental biology.

My investigations indicate that the growth of bacteria in artificial cultures is governed by the same laws as govern the development of a multicellular organism; that their cells during growth pass through exactly the same sort of a developmental cycle as the cells of a plant or animal, exhibiting in turn an embryonic form during the period of rapid growth, a mature or differentiated form during the period of slow growth or rest, and a senescent form during the period of death; that in short we may speak of a "cytomorphosis" in populations of free unicellular organisms differing only in degree from that of muticellular individuals. If this hypothesis be accepted it must prove of great importance in general biology, for it carries with it the implication that multicellular organisms are after all but

populations of cells, whose organized individually is a result, not a cause, of the differentiation of those cells.

These researches have been supported by grants from the Graduate School of the University of Minnesota, for which I am deeply grateful. I wish also to express here my gratitude to the Department of Dairy Industry of Cornell University, which has provided stenographic assistance in the preparation of the manuscript; to Dr. W. P. Larson, head of the Department of Bacteriology at the University of Minnesota, who has made every effort to provide the special equipment necessary for my work; to Dr. J. M. Sherman, who has kindly read the manuscript of Chapter I; and to Dr. Otto Rahn, who has similarly read Chapter II for me. Above all I must acknowledge my obligation to Dr. R. E. Scammon, of the Department of Anatomy of the University of Minnesota, without whose suggestions this work would not have been undertaken, and without whose kindly assistance and friendly criticism it could not have been carried through.

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CHAPTER I

THE PROBLEM OF MORPHOLOGIC VARIATION OF BACTERIA

Bacteriology has developed more as an applied science than as a theoretical one, and the fundamental biological problems of the bacteria have been neglected. There are many reasons for this, but perhaps one of the most important has been the blind acceptance by the majority of bacteriologists of the Cohn-Koch dogma of the constancy of cell forms and the immutability of bacterial species, which has discouraged all investigation of problems of morphology, inheritance, and variation in bacteria for a good many years. The progress of science is measured not so much by the wealth of fact established as by the veins of unsolved problems uncovered, and the moment a field of knowledge comes to be considered complete, at that moment this branch of science is dead. It is therefore a healthy sign that within recent years, through the activities of a small but persistent group of modern pleomorphists, bacteriology is definitely breaking away from the Cohn-Koch tradition and is seriously reopening for discussion and investigation the old problem of morphologic variation in bacteria.

But this movement has within it that danger inherent in all revolutionary movements, the tendency to go too far in the opposite direction. It does not necessarily follow that because Koch was wrong Naegeli was right; and yet in the writings of at least some of these new pleomorphists, their protests to the contrary notwithstanding, there is clearly evident a tendency to return to the old idea that any bacterium may transmute to the form of any other. Von Niessen, referring to Löhnis' work, exclaims, *"Im Prinzip Naegeli redivivus, Bestätigung deiner Befunde, völlig unabhängig und rein wissenschaftlich, und—was wird die führende Kochshe Schule dazu sagen?"* and indeed the dominant school of bacteriologists has so far had very little to say. A hypothesis endlessly repeated without criticism becomes a dogma, and it is high time there-

fore that we stop to evaluate the data and critically analyze the logic of these disciples of the "newer biology" of bacteria.

While occasional contributions have been made by a number of other authors, the principal members of this modern school of pleomorphists are Almquist, Hort, Löhnis, Mellon, and Enderlein. It is noteworthy that, excepting Almquist, all of these men made their initial contributions to the subject in the years 1915-1917. For details of their work the reader must consult their published papers, the most important of which are listed at the end of this work. But we may with profit summarize briefly here the ideas of each concerning the nature of the morphologic variations of bacteria.

Almquist has made observations of various members of the colon-typhoid-dysentery group of organisms, especially in old cultures kept at low temperatures on drying media. In his earlier work he has particularly emphasized the formation of exogenous globular forms which are produced from the normal rod forms by lateral or terminal budding. If these give rise again to rod forms directly they are called conidia; if they form new globular forms endogenously they are sporangia, and the cells so formed are gonidia; this process, however, may result from a sexual conjugation in which case the mother cell is an oöspore, the male element being a very small conidium called an antheridiospore. From cultures containing these conidia a new type which bred true was separated by filtration through Berkefeld filters, the obvious conclusion being that the cells which passed through were minute conidia (1911). These new types when used as antigens gave rise to agglutinins for the parent culture. In later papers (1924) he claims to have proved the occurrence of sexual reproduction by the production of hybrid crosses between typhoid and dysentery organisms, some of the colonies from mixed cultures of these two organisms being agglutinated by both serums; and by the discovery of haploid and diploid phases in the nuclei, the diplonts being large globular forms with a nucleus twice as large as in the normal, haplont rod forms.

Hort, believing that all of the phenomena observed in epidemic cerebrospinal meningitis could not be explained by the cur-

rent views as to its etiology, was led to make some filtration experiments, and found that from filtered urine, blood, and cerebrospinal fluid from cases of meningitis, there could be cultivated a pleomorphic organism appearing as large and small rods and cocci (1915). He concluded that the meningococcus is but a late non-virulent stage in the life cycle of an organism which has a filterable, virulent phase. Microscopic observation of cultures of meningococci revealed great variation in size and the formation of small cells from larger ones by budding, also refractile globular structures were found forming within some of the larger cells which he says are ascospores. He therefore considers the meningococcus to be a pleomorphic fungus belonging to the ascomycetes (1917a). Multiple lateral and terminal buds were also found to develop on typhoid and dysentery bacilli grown in acid media (1917b), and these were observed to give rise again to normal rod forms (1920).

Löhnis (1916) observed a great variety of cell forms in cultures of *Azotobacter,* and particularly the occurrence of slender, spore-forming rods, from which he concluded that the normal morphology of this organism is but a stage in the life cycle of a spore forming bacillus. Further study of this organism led to the conception of a complex cycle which was extended to apply to all bacteria by observations of some forty-two other species. Later (1921) appeared a very comprehensive monograph, which in spite of its bias is an extremely valuable review of the literature, in which he showed that the structures described by him had been repeatedly observed, described, and illustrated by many of the previous bacteriologists, among them the most orthodox.

Löhnis' hypothesis is so complicated that it cannot be readily summarized. A few quotations may serve to outline its general scope.

> Contrary to the monomorphistic theory which only knows one constant type of vegetative cell for every species, and not more than one type of reproductive organ, the endospore, for merely one group of bacteria, it has been ascertained by numerous independent investigations that in reality all bacteria are not only distinctly pleomorphous in their vegetative growth, but are also able to produce various organs of reproduction. These are: Gonidia, regenerative bodies, exo- and endospores, arthrospores, and microcysts. All of them are made up of nuclear substances, which are reinforced by smaller or larger amounts of

reserve material and protected by a more or less resistant membrane. Gonidia and regenerative bodies participate actively in the process of multiplication, whereas the other reproductive organs are in the first place resting forms. Gonidia, regenerative bodies, and probably microcysts are produced by all bacteria, while arthrospores, exo- and endospores are less common; though there are indications that they will be discovered in many more cases, as soon as these problems are more thoroughly investigated (1921, p. 162).

All bacteria live, in vivo as well as in vitro, alternately in an organized and in an amorphous stage. By the partial or complete dissolution of the vegetative and reproductive cells, a plasmatic mass, the symplasm, is formed, which, after a period of rest, according to circumstances may transform itself into new cells of the same or of a more or less modified character. . . . . The formation of the bacterial symplasm proceeds always in two phases: First, the cells agglomerate to smaller or larger clumps; second, a more or less complete dissolution of the cells takes place, resulting in a crumbly or slimy mass. . . . . The reconstruction of new cells from the bacterial symplasm follows various lines according to the internal as well as the external conditions (quality of the symplasm, environment, and technique). At first always regenerative units become visible, which either grow up separately to new cells, or of which several may agglomerate and surround themselves with a uniting membrane, thus forming at once full-grown cells. (1921, p. 195).

He also describes another process as conjunction, where two or more cells combine without previous disintegration. Löhnis is careful to point out that "by discussing the life cycles of bacteria we do not intend to revive any of those unclear theories concerning bacterial polymorphism or pleomorphism. The development of bacteria is characterized not by the irregular occurrence of more or less abnormal forms but by the regular occurrence of many different forms and stages of growth connected with each other by constant relations" (1916, p. 677). But the diagram which he presents to show these constant relations, with its numerous arrows connecting each type with many others, and with double points indicating their reversibility, leaves one without any clear notion of regularity, but rather with the idea, in vogue in Naegeli's day, that any bacterial type may develop in a hap-hazard way from any other. Indeed, from neither text nor illustrations can one gain any exact impression of the sequence of events in the life cycle he postulates.

Mellon, in a long series of papers extending over ten years, has described morphologic variations in diphtheroid bacteria, typhoid and colon bacilli, and fusiform organisms; many of his papers deal with

variations in serologic or cultural characters rather than morphology, but the origin of the former is traced to the latter in every case; some of his work has had to deal with pathogenic fungi rather than bacteria. Again the work is too voluminous to be readily summarized in a few words. But the main idea seems to be, as in the case of Löhnis, that bacteria go through a more or less complex life cycle in which various types, rods, spheres, branching forms, etc., appear in sequence; further, that any one of these types may be stabilized, i.e., continuously reproduce itself, without going through the rest of the cycle. The origin of these stabilized types is sought in sexual reproduction, which is found particularly in the case of the colon bacillus in the formation of large globular swellings, previously described and illustrated by Scales, at the point of fusion of two rod forms, and designated by Mellon as zygospores, though conidia and even endospores are thought to involve at least a process of autogamy. Mellon believes that the name applied to these structures is relatively unimportant as long as the essential factors of conjugation and nuclear reorganization are recognized. He also describes the separation of variants from cultures by filtration which differ markedly from the parent strain, especially in virulence, and implies, as have the preceding authors, that these variants have been derived from minute, perhaps ultramicroscopic, conidia.

The latest, and perhaps most remarkable contribution to this new pleomorphism is the work of Enderlein. He unfortunately has found it necessary to coin a vocabulary of nearly two hundred new words to express his ideas, which proves a serious obstacle to the reading of his work. Once this vocabulary is mastered, however, one finds that the hypothesis is presented with a clearness and coherence that is annoyingly lacking in the papers previously reviewed. I say hypothesis, for one gains the impression that the ideas expressed are pure theory having only the most slender basis in actual observation. Only slight details of technique and brief descriptions of actual observation are given. The drawings, made with extremely high magnifications, are not convincing. His description of the centrosome with dimensions of 0.01μ, which, as is well known, is quite below the limits of resolution of the microscope, seriously impairs one's faith in the accuracy of his other observations. This impression

that the work is a philosophical one rather than the result of laboratory observation is supported by the fact that the author is a zoölogist who has made no other contribution to bacteriology.

Enderlein considers the cytology, life cycles, and taxonomy of bacteria. The basis of his cytology is the primitive nucleus or "mych." This with its accompanying protoplasm forms the "mychit," which is the building stone of bacterial cells. It may exist by itself as in the micrococci, or may combine with others in various multiples to form "pliomychits," the cells of larger or more complex bacteria. Asexual reproduction is accomplished through equational cell division, "monogony," in the case of a mychit; "isomorphous arthrogony" in the case of more complex cells; or by unequational cell division, "heteromorphous arthrogony," which can occur only with pliomychits. The latter may give rise to a number of different reproductive bodies: Gonidia, which contain a single mych; the "cystit," a gonidium with a compound nucleus ("polydynamen mych"); the "cystoid," an unusually large gonidium with much reserve foodstuff; structures behaving like embryos in that they grow at the expense of a neighboring or enclosing mother cell; and structures like the oidia of higher fungi formed by the multiple fragmentation of a cell. Sexual reproduction is accomplished by the development of gametocytes from gonidia. These undergo a reduction division of the mych, the haploid mychit resulting being called a "gonit." The latter may develop into a male cell ("spermit") or a female cell ("oit"). The former, in the case of the cholera vibrio, is smaller, curved, and actively motile by means of a polar flagellum; the latter is larger, globular, and but sluggishly motile. These unite, followed by a fusion of their respective nuclei, giving rise again to the diploid phase. Enderlein also confirms Löhnis' observation of the symplasm.

The life cycle of a bacterium, according to Enderlein, consists of two simultaneous, parallel, and co-ordinated processes, a multiplicative development through simple cell division, and a progressive development ("*fortschreitenden Entwicklung*"), very slow and characterized by morphologic variation. The former is "auxanogeny," the latter "probæogeny." As the ontogeny of an individual repeats the phylogeny of the race, so the life cycle of a bacterium repeats the evolution of the species; and as a multicellular organism cannot avoid

in its life cycle of development the one-celled stage, the egg, so the bacteria are derived from and return to the elementary unit, the mychit. Each complete life cycle begins with the fusion of two haploid mychits, goes through progressive changes in cell complexity, reaches a maximum fixed for the species, and returns again to the haploid mychit. But cycles may be incomplete, shortened, or completely arrested. The progressive development may be completely eliminated and only the multiplicative may occur, and this condition, "mochlosis," is usually the case when bacteria are maintained in a uniform environment as is true when artificially cultivated in the laboratory.

Space will not permit of a more complete review of this remarkable work, but enough has been given to indicate that, while the whole thing sounds like a strangely complex product of the imagination, the hypothesis is a very orderly and systematic one compared with the rather vague and confused ideas of Almquist, Löhnis, Mellon, and Hort.

The work of Bergstrand should be noted here, though it should not be considered in the same class with that of the authors previously mentioned, since he does not draw such radical conclusions. From a very extensive and careful study of morphologic variations of a diphtheroid organism (1919), he concludes that morphologic variations of bacteria are much more extensive than is generally believed, and is opposed to the idea that all variations from the normal are to be considered involution or degeneration forms. Bacteria may reproduce by budding or by abstriction. The pleomorphism of bacteria may probably be explained by the fact that they are fungi which may form mycelium. He considers the large globular forms produced by some bacteria, identified as sexual forms by Mellon and Almquist, as resting cells analogous to or identical with the chlamydospores of higher fungi (1923). He finds no evidence of sexual spore formation (1923) or of a nucleus (1921).

Bergstrand does not go nearly so far as the others, but is satisfied to emphasize the extent of the variation which occurs and the importance of the problem. The reader will bear in mind, therefore, that most of the criticism which follows does not apply to his work.

Notice should also be taken of the papers of Fuhrmann on the

life cycles of bacteria. He recognizes two cycles, a minor one consisting of a single generation, i.e., the changes involved in the development of a cell from one division to the next, and a major one consisting of the regular changes occurring during the growth and aging of a culture. This conception was developed from observations of morphologic variations of an organism isolated from beer, *Pseudomonas cerevisiæ*. The young forms are motile rods; with increasing age of the culture these tend to lose their motility and to form chains; with still greater age certain of them become swollen at one end and accumulate numerous metachromatic granules. These latter *dauerformen* are the more resistant to various agents than are the young cells, but not as resistant as the endospores of spore-forming bacteria. With the latter the endospore formation occupies the same stage of the major cycle as do the *dauerformen* of the beer organism. When the latter are transferred to fresh media they rupture and discharge several small cells which immediately develop into motile rods. He is rather inclined to believe that the metachromatically stained granules of the *dauerformen* are of a nuclear nature, and that the *schwärmer* develop from them.

Fuhrmann lays considerable stress upon the regularity with which the various changes in morphology occur in succession during the aging of a culture. Various external factors tend to influence this cyclical development only in degree. The age of the culture seems to be the most important factor. To this extent his ideas anticipate somewhat and are in agreement with my own. His papers have only come to my attention during the preparation of this manuscript.

While in most of these papers no exact details of the nature and sequence of the supposed life cycles are given, and the various authors are not in perfect agreement on all points, the same general idea clearly runs through all of their writings. This idea has perhaps been most clearly formulated by Mellon, who states that "bacteria in their fundamental biology are in reality fungi that have been telescoped down, as it were, to a somwhat lower order; but this order is not so low as to preclude the preservation by the bacteria of the fundamental organization characterizing the fungi and higher plants. . . . . In point of fact, instead of reproducing themselves by transverse fission alone, we know that under conditions none too specialized

bacteria may bud, branch, form gonidia, and even structures comparable with asci. The latter characteristic implies a complicated life cycle sexual in nature. . . In any event, it is clear, and now beyond dispute, that Koch's law of morphologic type specificity cannot longer be regarded in the absolute sense which has characterized it in the past. Bacteria do change their morphologic type and within very wide limits; and with this change may go at times important physiological modifications" (1927, p. 107).

Much can and will be said in criticism of both the work and the hypotheses of these men, but this criticism should not in any way minimize the real value of the service which they have rendered to bacteriology in reopening for discussion and investigation this fundamental problem. Confronted with the cold skepticism towards questions of morphologic variation in bacteria common to bacteriologists a decade ago, it required real courage to publish papers of the type here reviewed. And while these papers have probably not convinced more than a very few that bacteria exhibit complex fungoid life cycles, they have demonstrated beyond question of a doubt that bacteria do regularly show pronounced morphologic variations, the nature and significance of which must be determined before we can make any real progress towards understanding the fundamental biological problems of the group.

One can no longer satisfactorily answer their arguments by the old formula that they are dealing with impure cultures; for while undoubtedly in isolated cases conclusions have been drawn from observations of contaminated cultures, this is by no means general, and anyone who will patiently study with the microscope his own cultures which he knows to be pure can quickly confirm the general observation that rod forms may appear in cultures of cocci, spherical forms in cultures of bacilli, lateral buds and branches, and internal globular bodies in both. In undertaking a critical analysis of this work, then, one cannot find fault so much with the actual data as with the logic followed in erecting the hypothesis.

But the data are not beyond criticism. Throughout these papers one is impressed with the meagerness and haphazard character of the observations as compared with the widespread importance of the conclusions. The small amount of data submitted may per-

haps be explained in part by the present day difficulty in obtaining sufficient space in scientific journals for complete reports; but the descriptions of observations offered do not give a hint of any wealth of observation withheld from publication. The observations are for the most part casual, not the result of systematically planned experiments. Cultures are examined today and then set in the icebox for weeks or months, at the end of which time they are found to have undergone a transformation; but a systematic step by step observation of this transformation with a record of all the transitional stages is lacking. Enderlein states in criticism of the monomorphists, "Der methodische Hauptfehler war anfangs im Hinblick auf die Cyclogenie der Mangel einer methodische Beobachtung der Veränderungen im Laufe der Zeit; meist wurde nur mit jungen Kulturen gearbeitet." But if one substitutes "old" for "young" in this statement it applies equally well to the pleomorphists.

The failure to make continuous observation and record of all stages of the transformations described is the most serious criticism of the factual material in the work under discussion. It is due to the technical difficulties involved; but certainly much more could have been done in this direction than has been recorded. It is only fair to state that a certain amount of continuous microscopic observation has been recorded: Hort (1917a) has illustrated the formation and separation of buds and the formation of the intracellular structures which he calls ascospores in meningococci, and the formation (1917b) and later development into normal rods (1920) of buds in typhoid bacilli; Almquist records the transformation of spherical conidia by germination into a "probacterium" in typhoid bacilli; Enderlein claims to have followed the fertilization of the "oit" by the "spermit" in cultures of the cholera vibrio. Gardner has clearly observed the origin of normal rods from branched cells, without, however, concluding that they form part of a complex life cycle. But these isolated cases only serve to emphasize the general lack of that continuous step by step observation which must be made before any clear idea of a life cycle can be established. It should also be stated in all fairness that frequently it is impossible to tell from the author's statement whether in particular cases he is recording hypothesis or stating observed fact; statements of hypothesis are made so positively

that they sound like established fact, particularly in the works of Löhnis and Enderlein. I have assumed in such cases where illustrations or actual descriptions of the method are not furnished that the statement has not been verified by actual continuous observation. In most cases, however, it is quite clear that no continuous observation has been made, and that the author's idea of the sequence of events has developed from an attempt to trace the transitions in different individuals in the same preparation. This clearly involves an element of error. The structures, for instance, which Mellon interprets as zygospores, namely two cells with swollen ends in contact, might just as well be interpreted as a cell with a central bulging undergoing division.

This lack of systematic observation, this attempt to patch together the life cycles from haphazard observation of cultures in widely different media without regard to the age of the culture or the phase of growth, is responsible for the vague and incoherent nature of the descriptions of the life cycles presented. In practically no case can one obtain from the published papers any clear notion as to the author's ideas regarding the actual sequence of events in the cycle; as to when gonidia are formed, and when asci and zygospores; under what conditions simple conjunction occurs and under what circumstances a symplasm results. Löhnis' complicated diagram is absolutely unintelligible, and the one published by Mellon to illustrate the ontogeny of a diphtheroid organism (1926, v), while simpler, is far from being clear.

The fact that practically all of these observed variations in morphology have been found in old cultures, and especially on unusual or unfavorable media, of course at once leads one to believe that they are "involution forms" i.e., dead or degenerating cells. This argument is answered at once by the statement that the cultures containing them are perfectly viable. But the fact that growth occurs when such old cultures are transferred to a new medium does not prove that this growth has resulted from the cells showing the morphologic variation, even when such variant cells preponderate. Only careful quantitative observation of the proportion of viable cells and of morphologically variant cells, or single cell isolations of variants to new media, or continuous observation of particular variant cells trans-

ferred to new media will answer this question of viability. None of the authors has followed the first procedure; Mellon has used the second in a few instances, and as has been mentioned above, Gardner, Hort, and Almquist have followed the third in the case of budding and branching cells.

Hort states that an involution form is, "strictly speaking, a sterile organism which is not only incapable of maintaining its reproductive activity, but is also incapable of maintaining its integrity of form" (1920, p. 370). Whatever may be the dictionary definition of the word, this idea is erroneous, for senescence and death are processes requiring some time and the terms are relative. It may well be that an injurious agent or process has led to the deformity of a cell long before it has actually been killed, and that such a cell, an involution form in the sense that it has undergone a modification of form as part of a degenerative process, is still capable of "reviving" and multiplying when transferred to a more favorable environment. Even the proof of viability of single variant cells does not, therefore, prove that their variation is not the result of a degenerative process.

Throughout these works is the assumption that bacteria possess discrete nuclei; and Almquist, Mellon, and Enderlein have definitely interpreted structures which they have seen as nuclei. It would take too much space to debate here this much disputed question concerning the presence or absence of nuclei in bacteria, but I feel safe in stating that no one has so far demonstrated certainly that any of the true bacteria possess a discrete nucleus. The structures described by Meyer and Paravicini are the only ones that have been reported which fulfill the requirements of a nucleus, but their findings have not been confirmed by anyone except in the case of endospore-forming bacteria during the stage of spore formation. The assumption that bacteria possess nuclei is therefore unwarranted, and the casual way in which this or that intracellular structure is designated a nucleus without any clear evidence is characteristic of all of the data. Almquist mentions no attempt to differentiate his nuclei from volutin, neither does Mellon, though Meyer has clearly pointed out the danger of mistaking this material for chromatin and the means of differentiating it. Mellon lays stress on the skein-like structure as evidence of a nuclear nature, but this structure may be

assumed by volutin in old cultures of diphtheroid bacilli (personal observation). Löhnis accepts the older uncritical works on the cytology of bacteria, and almost any internal structure may be a nucleus. Enderlein claims to have differentiated his "mych" from reserve foodstuffs, but I doubt that his observations can be substantiated. Almquist's recognition of haploid and diploid phases on size alone hardly deserves serious consideration. In this connection it should also be noted that Löhnis has apparently made no attempt by micro-chemical methods to determine the nature of his regenerative granules, i.e., whether or not they might be volutin, and Hort has similarly overlooked this possibility with regard to the so-called ascospores of the meningococci. In short such differential staining processes as are at our disposal for identifying intracellular ergastic substances have not been used on the various structures interpreted as nuclei, gonidia, regenerative bodies, etc., and until they have been used we are justified in suspecting that these structures may be fat or volutin or other material.

The most serious criticism of this new pleomorphism, however, has to do not so much with the data or the technique, as with the line of reasoning which has led to the development of the theories of life cycles. Nowhere does one find a clear argument starting from proved facts, but only either vague analogies or conclusions drawn from unwarranted assumptions. An example from Almquist will serve to illustrate the most important fallacy. He says:

> Die männlichen Antheridien habe ich zweifelsohne oft in meinen Präparaten gesehen, obgleich ich die Kopulation nie sicher beobachten konnte. Wohl sah ich manchmal geschwollene Formen mit feinsten, an der Seite festsitzenden Körnchen oder Nädelchen. Dem näheren verlauf ich konnte aber nicht folgen. Ich nehme doch an, dass die zahlreichen, 0.5μ oder darunter messenden Mikrokonidien und auch andere feine Körnchen zu den Antheridien gehören.

Henceforth these structures are referred to as antheridia, though their identity as such is only an assumption on the part of the author. The same thing has been done repeatedly by Mellon, Löhnis, Hort, and Enderlein, though never so frankly admitted. Structures *look like* conidia or ascospores or zygospores or what not; therefore they are named such and in further reasoning are considered as such,

as though the mere naming of them had somehow or other proved the author's opinion as to their identity.

This persistent naming of all the structures described with names borrowed in part from mycology, in part from cytology, or as in the case of Enderlein, coined for the occasion, only serves to obscure the problem and to make it more difficult for the reader to follow the author. One cannot help but suspect that the necessity for using a new terminology has its origin in the obscurity of the thought which the author is trying to express. The confusion introduced by this terminology is all the greater because the same structures are referred to by the various authors under different names, and the same names are used to designate different structures. Thus the lateral and terminal projections from cells are referred to by Hort simply as buds, by Almquist as conidia, by Löhnis and Enderlein as gonidia, by Mellon sometimes as conidia, sometimes as exospores. Almquist speaks of a single bacterial cell of irregular or ameboid form as a plasmodium, but states that this is identical with Löhnis' symplasm; while Mellon applies the same term to the connecting filaments of protoplasm described by Meyer under the name plasmodesmids. Mellon refers to the globular structures arising in colon bacilli as zygospores, but to illustrate their nature offers for comparison a picture of the ascospores of Endomyces; and in a footnote implies that the two words have the same meaning. In fact, to Mellon the terms chlamydospore, arthrospore, *dauerzellen*, gonidium, and zygospore all apply to essentially the same sort of a process; "they may be viewed as branches of the same reorganization tree." But these terms have very distinct meanings in mycology, and refer to structures physiologically as well as morphologically different, and their confusion can only result from an imperfect knowledge of the nature of the processes under consideration.

There is another unwarranted assumption in the reasoning of these various authors, namely that permanent variation is an evidence of sexual reproduction, that all variants are hybrids. No matter how well we may consider this to be established for higher organisms, we are certainly not justified in accepting it for unicellular organisms where non-sexual reproduction is common and where there is no differentiation into somatic and germ cells. Jennings has shown that

by artificial selection quite different races may be obtained from cultures of protozoa in which no sexual reproduction has occurred. No clear evidence of the occurrence of hybridization in bacteria has as yet been furnished, Almquist's experiments with so-called typhoid-dysentery crosses being too incomplete. It is not enough to show that variation has occurred; it must also be shown that the variation follow the Mendelian law. This has recently been claimed for the *mutabile* variant of the colon bacillus by Stewart, but his article is not convincing since it was possible to obtain from the "hybrid" only one of the assumed parent factors. His assumption that the other character was associated with a lethal factor that suppressed growth is hardly warranted, since the same conditions hold with practically all bacterial mutants, and it is hardly likely that in every hybridization one of the characters is associated with a lethal factor. Topley and Ayrton have pointed out that these variations can be as well explained by a non-equational cell division; that we may have segregation without syngamy. With most bacterial variations it is rather difficult to distinguish between temporary modifications and true mutations, and the possibility of inheritance of acquired modifications in microörganisms has not been disproved. In short, our knowledge of the mechanism of variation in unicellular forms is quite too incomplete to permit us to use such variations as a premise from which to argue the occurrence of sexual reproduction.

With these two unwarranted assumptions, that similarity of form indicates indentity of function, and that variation is necessarily an evidence of conjugation, we are led into a sort of circle of false logic which runs something like this: Certain cell forms of bacteria look like conjugating cells or products of conjugation. Let us call them such; they are ascospores (or what you like). Since they are sexual spores they should at times give rise to hybrids. Variations are found; therefore sexual reproduction occurs; therefore our assumption of the sexual nature of the observed structures was valid. But there is no proved fact anywhere in this argument, save the existence of both morphologic and cultural variation.

In fact, neither the data nor the logic of these new pleomorphists are adequate to convince us that bacteria possess complex fungoid life cycles. We may sum up the present status of the problem by

stating that bacteria in pure culture do show wide variations in the size and form and structure of their cells, but that the nature and significance of these variations are not known.

"Was ist nun zu tun?" says Almquist. "Wir müssen die Natur objektiv untersuchen und den Tatsachen unterordnen."

The problem is one particularly adapted to statistical analysis, since it deals with large numbers of individuals varying among themselves at any point in time, and varying continuously with time. This work has to deal with an attempt to apply such statistical methods to the problem of morphologic variation in bacteria, with the hope that by such methods the investigation will be truly kept objective, and that the facts will eventually truly be placed in order.

CHAPTER II

THE RATE OF GROWTH OF BACTERIA

". . . . It is perfectly true that all changes in form, inasmuch as they necessarily involve changes of actual or relative magnitude, may in a sense be properly looked upon as phenomena of growth; and it is also true, since the movement of matter must always involve an element of time, that in all cases the rate of growth is a phenomenon to be considered."

D'Arcy Thompson

The outstanding fact established by my studies is that bacteria are continually varying in morphology with increasing age of the culture in which they are growing, and that the morphologic variations are correlated with the rate of growth, the transition from one type to another occurring at the points of inflection between phases of the growth curve. Before discussing these morphologic variations, therefore, it is desirable to devote some space to a review of what is known concerning the growth curves of bacteria and the factors which determine them.

When we place viable bacteria in a suitable medium they generally do not commence to grow at once, but after a longer or shorter period of quiescence they begin to divide, at first rather slowly, then with greater and greater rapidity until a maximum growth rate is attained, after which they continue to divide more and more slowly until growth ceases altogether. After a longer or shorter period during which the number of cells remains practically constant, they begin to die, the rate of death becoming greater and greater until a maximum death rate is attained, then becoming slower and slower, some cells remaining viable in most cultures for long periods of time. Thus if we plot a curve of the number of living cells in the culture, against time, there is obtained a characteristic S-shaped curve of growth, which is repeated in reverse during the period of death.

Such growth curves for populations of microörganisms in cultures are essentially similar to the curves obtained for the growth

of other populations, as fruit flies in a bottle or human populations in a restricted area; and also to the growth curves of those co-ordinated populations of cells which we call multicellular organisms. This has been emphasized by Pearl (1925), who concludes that a common law governs the growth of all such populations, which he states as follows:

"Growth occurs in cycles. Within one and the same cycle, and in a spatially limited area or universe, growth in the first half of the

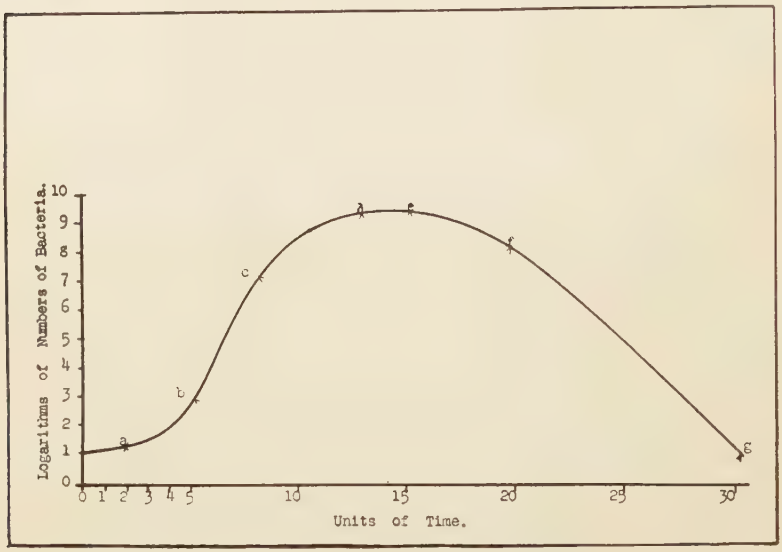

Fig. 1. The Growth Curve of Bacteria.

From "Life Phases in a Bacterial Culture" by R. E. Buchanan. Reproduced through the courtesy of the Journal of Infectious Diseases. (1918, 23, 109).

cycle starts slowly, but the absolute increment per unit of time increases steadily until the midpoint of the cycle is reached. After that point the increment per unit of time becomes steadily smaller until the end of the cycle. In a spatially limited universe the amount of increase which occurs in any particular unit of time, at any point of the single cycle of growth, is proportional to two things, viz.: (a) The absolute size already attained at the beginning of the unit interval under consideration, and (b) the amount still unused or

unexpended in the given universe (or area) of actual and potential resources for the support of growth."

Growth curves of bacteria are obtained by counting the number of cells in a culture by one or another method, usually plating, at regular time intervals during the growth of a culture. The results may be plotted in various ways. The actual number of cells may be plotted against time, as has been done in Fig. 9. This serves to show the absolute increase or decrease, but not the rate of change, and has the disadvantage that where the numbers are small, at the beginning and end, slight but relatively important changes are not shown. The usual method, illustrated in Fig. 1, is to plot the logarithms of the numbers of cells against time. Such a curve shows the proportional increase or decrease in the number of cells, and the slope of the curve is a measure of the rate of change; it possesses the advantage that variations at the beginning and the end, when the numbers are small, are plotted on a scale such that these variations are clearly distinguishable. Since, after the maximum growth period has passed, changes in the rate of increase or decrease occur at an increasingly slower rate, it is sometimes advantageous to plot the logarithms of the numbers of cells against the logarithms of the time intervals. This serves to magnify the scale of the abscissæ during the early stages of growth when changes are occurring rapidly. Such a curve cannot be used to measure the rate of growth, but still serves to show clearly the division into phases. It has been necessary to follow this procedure in Fig. 25 and Fig. 33 simply to obtain space for the cells forms and the growth curve on the same graph.

The rate of growth has also been expressed in terms of generation time, i.e., the time required for the cells to double in number. The mean generation time may be computed for any time interval by dividing the time by the number of generations, and the latter quantity may be determined from the cell counts at the beginning and the end of the time interval according to the formula

$$n = \frac{\log. b - \log. a}{\log. 2}$$

where n is the number of generations and a and b are the first and last cell counts, respectively. The mean generation times for the

various intervals of observation may then be plotted against time. Such a curve has the advantage that it expresses more clearly than the others changes in the rate of growth; it has the serious disadvantage that it tends to magnify small experimental errors in counting, so that most data must be first smoothed by some method before they can be used in this way.

Mathematical analyses of the growth curves of bacteria have been made by McKendrick and Pai, Ledingham and Penfold, Slator (1917), and Buchanan; of yeasts by Slator (1913, 1918, 1919), and by Carlson; of growth curves in general by Pearl and by Robertson; and various empirical formulæ have been derived to express these curves. Where these are not interpreted in terms of organisms, substrate, products of metabolism or other definite factors, they do not seem to be very helpful to an understanding of the phenomena to one who is not mathematically minded.

For purposes of description and analysis it has been generally found convenient to divide the growth curve of bacteria into phases. This is in a sense unfortunate in that it makes what is essentially a continuous process appear somewhat discontinuous, but is almost necessary for intelligent discussion. Lane-Claypon described four phases, an initial lag period during which the number of cells remains constant or very slowly increases, a period of maximum growth, a stationary or resting period, and a period of death. This phasing has been followed by most succeeding authors, but Buchanan further subdivided these phases, making seven in all, illustrated in Fig. 1, which is taken from his paper. They are the initial stationary phase, from the origin to *a,* during which the number of cells shows no increase (it may actually decrease, according to Chesney); the lag phase, or period of positive acceleration in growth rate, *a-b;* the logarithmic growth phase when the rate of growth is constant and maximum, the logarithms of the number of cells falling on a straight line, *b-c*; the phase of negative acceleration, *c-d*; the maximum stationary phase, *d-e*; the phase of accelerated death, *e-f;* and the logarithmic death phase, during which the cells are dying at a constant rate, and the logarithms of the number of cells again fall on a straight line, *f-g*. A final phase of negative acceleration in death rate might have been added, for in many cases at least the death

rate decreases after a time, and some cells may be found alive after relatively long periods.

This division into phases is more obvious in cultures which grow rather slowly. Most organisms when grown on the surface of agar develop much more rapidly than in liquid media (which have been used almost exclusively in growth studies of bacteria) and the growth curves of such agar cultures, as will be seen later, do not show such a sharp separation into phases, the so-called logarithmic growth phase being practically absent, the maximum rate being maintained for only a brief interval, so that the logarithmic curve is nearly S-shaped; this S-shaped curve is then repeated in reverse during the period of death, but extends over a much longer interval of time. The total growth curve thus has the appearance of a rather skewed frequency distribution curve. One can then distinguish a period of accelerating growth, a period of negative acceleration in growth, a period of accelerating death, and a period of negative acceleration in death.

Various factors, as temperature; the size, age, and previous history of the inoculum; and the composition and nutrient value of the medium, influence the form of the growth curves of bacteria. It is of course well known that growth is slower on either side of the optimum temperature, the decrease in growth rate being greater per degree with higher than with lower temperatures. It is apparent that the effect of temperature is due both to a shortening of the lag phase (Penfold, Chick) and to an actual increase of growth rate during the maximal growth period (Lane-Claypon, Barber). Lane-Claypon interprets the effect of temperature on the growth rate as an operation of the Van't Hoff law. Zikes (1919b) has made the interesting observation that the growth rate-temperature curve for yeast is modified by the temperature at which the strain has previously been cultivated. After being continuously subcultivated for some time at a low temperature (8 degrees), it was found that growth was more rapid at all lower temperatures than before, and that there was a tendency to develop two optima; this might be interpreted as indicating that the growth-temperature curve is in reality an expression of the frequency distribution of the cells with regard to their optimum temperature, and that by growth at low

temperatures a new strain with a lower optimum was being selected out. Graham-Smith has made the rather surprising observation that

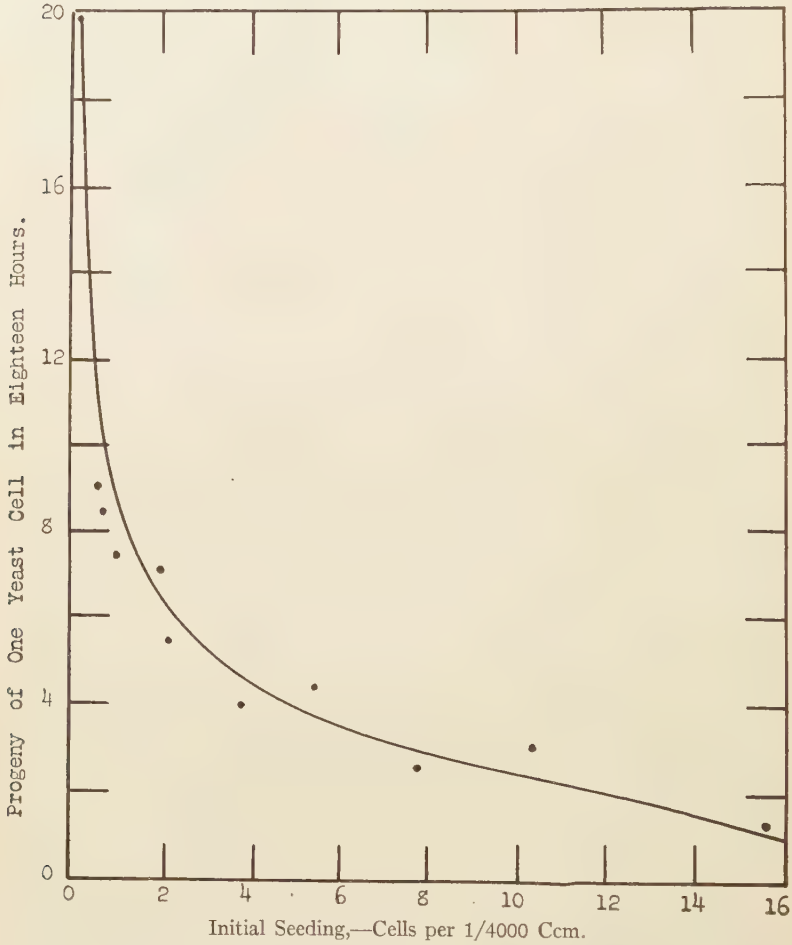

FIG. 2. INFLUENCE OF SIZE OF SEEDING ON RATE OF GROWTH OF YEAST. Plotted from data of Adrian Brown (Jour. Chem. Soc. Lond., 1905, 87, 1395.)

while growth is slower at lower temperatures, it continues longer and the final level of the culture is actually considerably higher

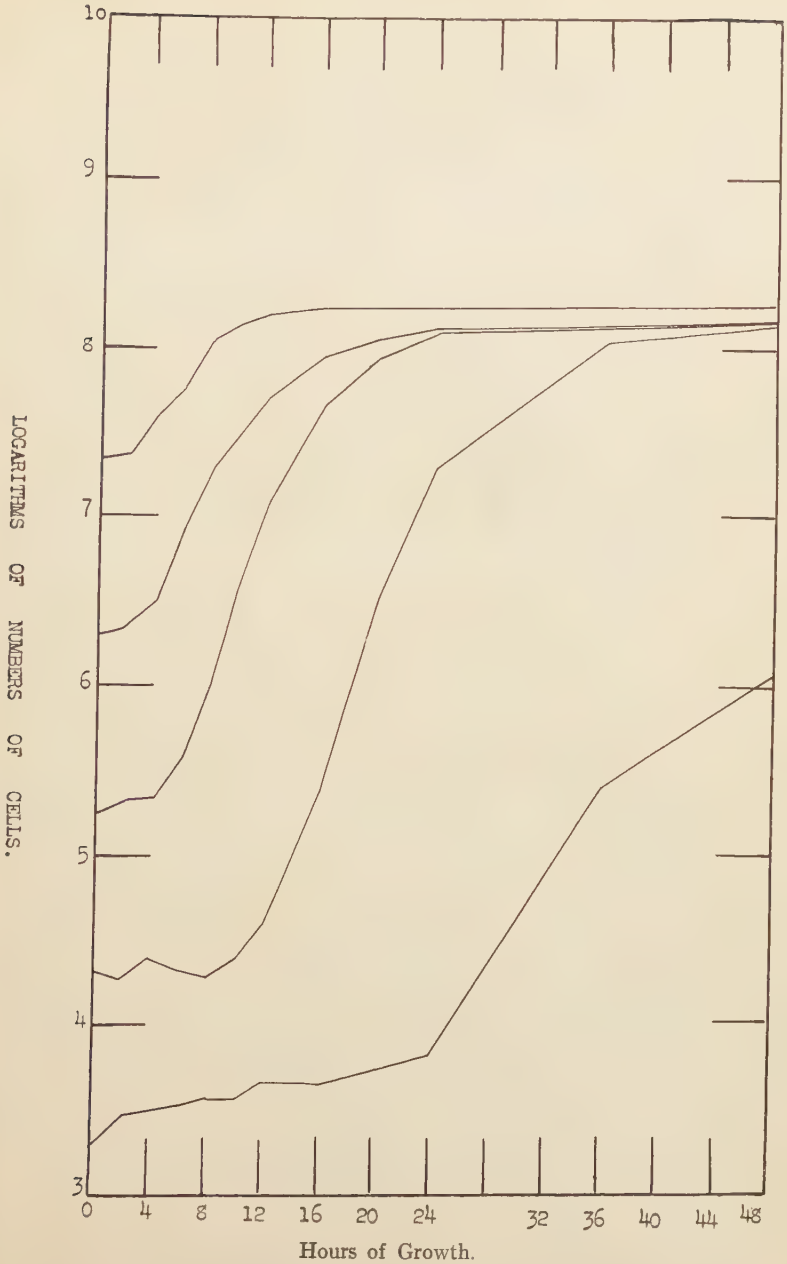

FIG. 3. INFLUENCE OF SIZE OF SEEDING ON GROWTH CURVES OF YEAST.

than in cultures grown at a higher temperature; the death rate is also correspondingly slower at lower temperatures. This was not confirmed by Tanner and Wallace with thermophiles.

Of the various factors which influence the rate of growth and the form of the growth curve, the initial number of cells introduced into a unit volume of medium seems to be one of the most important. Rahn first showed that the duration of the lag phase was greater with smaller seedings, and Penfold, although finding fault with Rahn's data, was able to confirm his conclusion. Montank has demonstrated the same thing for yeasts (Figure 3). It should be noted that the occurrence of a lag phase depends upon the age of the culture used for seeding, and that if the material used for inoculation is in the active growth phase no lag will occur in the subculture no matter how small the seeding (Sherman and Albus, 1926).

McKendrick and Pai noted that the final yield in cultures fourteen hours old was practically independent of the size of the seeding, and that therefore the rate of growth is higher for smaller seedings. This was confirmed by Graham-Smith as long as the seedings are smaller than the normal maximum yield of the medium. Adrian Brown has calculated the mean rates of growth of yeast cultures with various sized seedings, and derived a simple formula to express the relationship. Figure 2 is a curve plotted from his data, in which the yield from one cell in eighteen hours is plotted against the initial density of the population. It will be seen that the curve is hyperbolic, and that with very small seedings a very slight difference in the number of cells introduced makes a very great difference in the rate of growth, while with larger seedings the effect is slight. In these observations only the mean rate of growth for relatively long periods is available, i.e., it is impossible to separate the effects upon the lag phase and the growth phase.

Montank, working in my laboratory, has, however, obtained data in which such a separation is possible.* His results are shown graphically in Figure 3, the data for which are presented in Table I. In

*I am greatly indebted to Mr. Montank for the privilege of using here his as yet unpublished observations, part of an investigation still in progress. The data from which his curves have been plotted will be found in Tables 1 and 2.

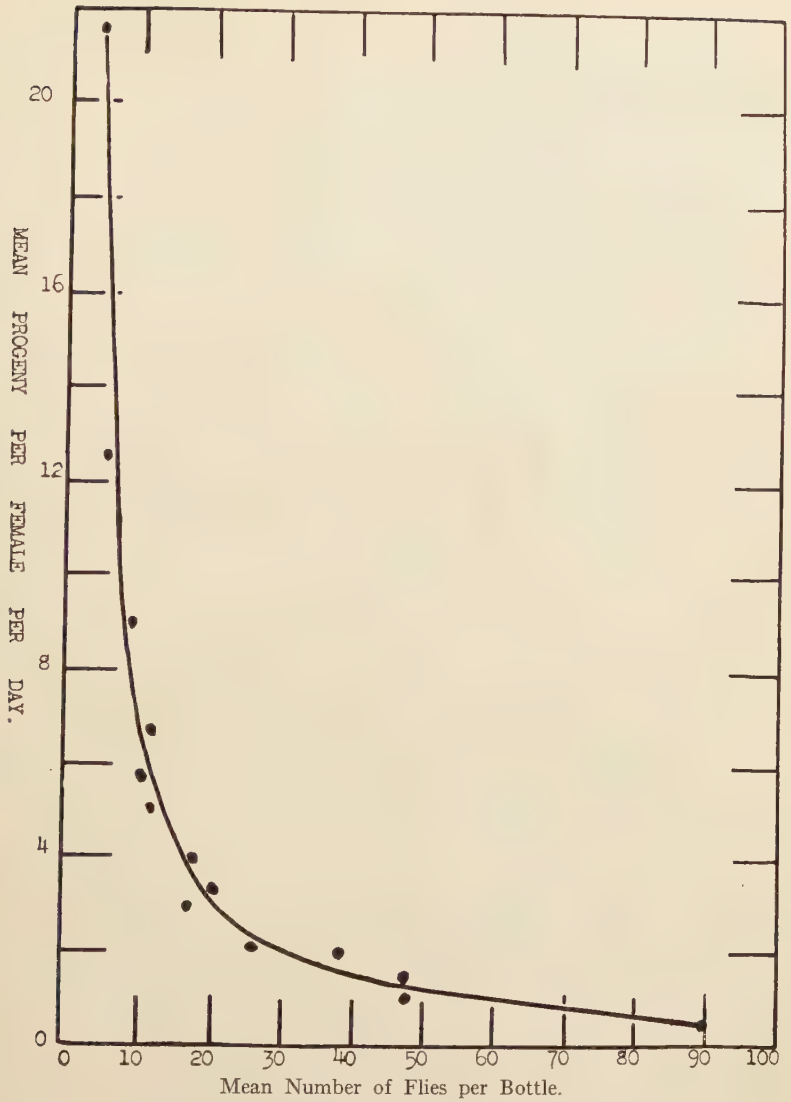

FIG. 4. INFLUENCE OF DENSITY OF POPULATION ON REPRODUCTION RATE OF FRUIT FLIES.

Reprinted from "The Biology of Population Growth" by Raymond Pearl, by and with permission of and special arrangement with Alfred A. Knopf, Inc.

these curves the logarithm of the number of yeast cells is plotted against time. It may be clearly seen that with smaller seedings, while the lag phase is prolonged in proportion to the decrease in the size of the seeding, in all cases (except with the smallest seeding), when once growth has been initiated, the actual rate of growth is higher, as indicated by the slope of the curve.

It is interesting to note that the size of the "seeding" has a similar effect upon the growth of individuals and populations of higher organisms. Figure 4 shows a curve for the mean rate of increase of a population of fruit flies from the data of Pearl. It is essentially the same as the curve plotted from Brown's data for yeasts (Figure 2). Figure 5 presents curves for the rate of growth of regenerating tadpoles' tails with different sized stumps, taken from the data of Ellis. The logarithms of total tail length have been plotted against time, and the graph is comparable with the curves for yeast growth in Figure 3. It will be noted that the same relationships hold. With a large stump (seeding), only a small part of the tail having been removed, there is no lag, and the rate of regeneration is slow, as indicated by the slope of the line; with decreasing size of the stump, the rate of regeneration becomes higher, and with the smallest there is a distinct lag before regeneration commences.

The age of the culture from which the inoculated cells are derived also has an influence upon the rate of growth in the new medium. Müller first demonstrated that the duration of the lag period was greater with older cultures. While Rahn and Coplans found that this was true, they still observed some lag when the culture used for seeding was young, and concluded that the lag phase could not be entirely eliminated by using actively growing cells. But Barber, isolating single cells, found that if these were growing at their maximum rate when transplanted, they would continue to grow at that rate in the new medium, and Penfold observed that the same thing was true with ordinary culture transplants, pointing out that probably Coplans and Rahn were dealing with cultures which had passed the maximum growth phase even though they were young as measured in hours. Chesney has demonstrated this effect of age of the parent culture most clearly in his work on the lag phase of the pneumococcus. Figure 6 is a graph copied from

his paper, the lower curves showing the initial rates of growth of subcultures from the parent culture, the growth curve of which

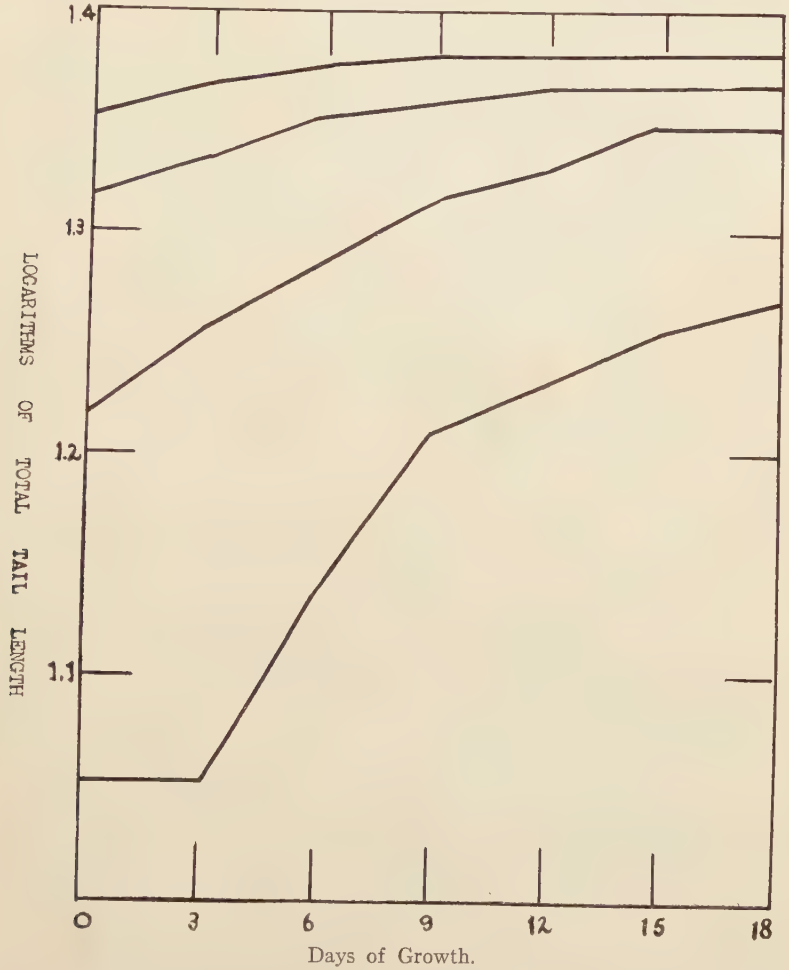

FIG. 5. INFLUENCE OF SIZE OF STUMP ON RATE OF REGENERATION OF TADPOLES' TAILS.

Plotted from data of M. M. Ellis (Jour. Exp. Zool., 1909, 7, 421).

is shown immediately above. It will be noted that when subcultured during the lag phase, the subcultures show no immediate

growth, but when subcultured during the logarithmic growth phase, they grow at once at the same rate as in the parent culture, while after the resting phase has been reached in the parent culture, the subcultures show slight or no growth during the first two hours.

The previous history of the culture used for seeding apparently has some influence upon the rate of growth or the form of the growth curve. Penfold states that transfer from one medium to

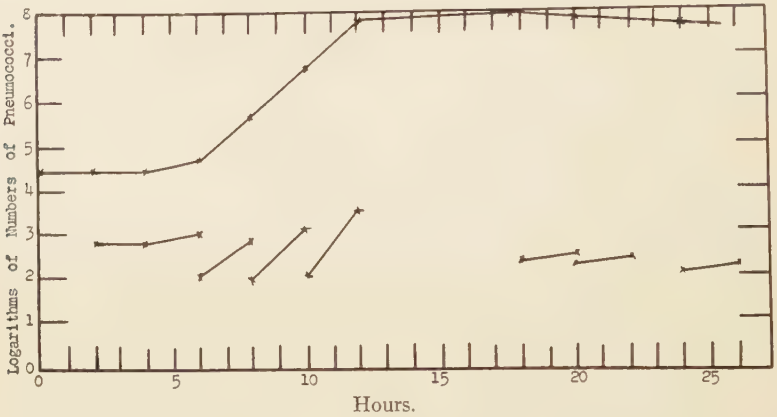

Fig. 6. Influence of Age Of Parent Culture on Initial Growth Rates of Pneumococci.

From "The Latent Period in the Growth of Bacteria" by A. Chesney. Reproduced through the courtesy of the Journal of Experimental Medicine. (1916, **24**, 387).

another of different composition may give a longer lag, the organism requiring a period for adaptation to the new environment, although he presents no data of his own. I have had an occasion to test this theory, having at hand two cultures of the colon bacillus derived from the same parent culture which had been carried for over five years continuously, one on ordinary nutrient agar, the other on a "synthetic" medium composed of various salts and glycerine. These were subcultured simultaneously into flasks of nutrient broth and the above mentioned medium, taking care to keep the size of the seedings approximately the same. While the growth rate was somewhat higher in the case of the broth cultures, both strains behaved approximately the same, and there was no appreciable

difference in the duration of the lag phase. Graham-Smith has observed that the frequency with which the parent strain has been subcultured influences the rate of growth, the growth rate being slightly higher and the final yield somewhat greater if the parent culture has been rapidly transplanted for some time.

McKendrick and Pai concluded from their mathematical analysis of the growth of bacteria that the concentration of nutrients in the medium was a limiting factor for growth, and that the rate of growth was proportional to this concentration, and the same conclusion was arrived at by Carlson for yeasts. Penfold and Norris observed the effect of varying the concentration of peptone on the rate of growth, taking care to eliminate the lag phase from their data. Their results are presented graphically in Figure 7. In reading this graph it should be noted that the ordinates are the generation times, the reciprocal of the actual rate of growth. With this in mind it is interesting to compare their curves with that of Brown for the effect of the size of seeding in yeasts. The two curves are very similar. It would seem, therefore, that the amount of available foodstuff bears a reciprocal relation to the number of cells in its effect on the growth curve. With small concentrations, slight differences have a profound influence on the rate of growth; with larger concentrations the effect is much less, the critical concentration in this experiment being about 0.4 per cent peptone. Salter, using higher concentrations of peptone, also found that increasing the concentration increased the rate of growth, the effect being most pronounced in the lag phase. Curran, on the other hand, found very little difference in the rate of growth in cultures in 1 per cent and 4 per cent peptone, and this difference was manifested in the later stages of growth, not at all in the lag phase. Zikes (1919a) also found that while the final yield of yeast was proportional to the concentration of wort, and the rate of growth must be correspondingly proportional, the initial rate of growth was actually somewhat greater with smaller concentrations of foodstuffs. Montank has observed the rate of growth of yeast in media of decreasing concentrations of dextrose and peptone. His results are shown graphically in Figure 8, the data in Table II. There was practically no effect on the duration of the lag phase, but the rate

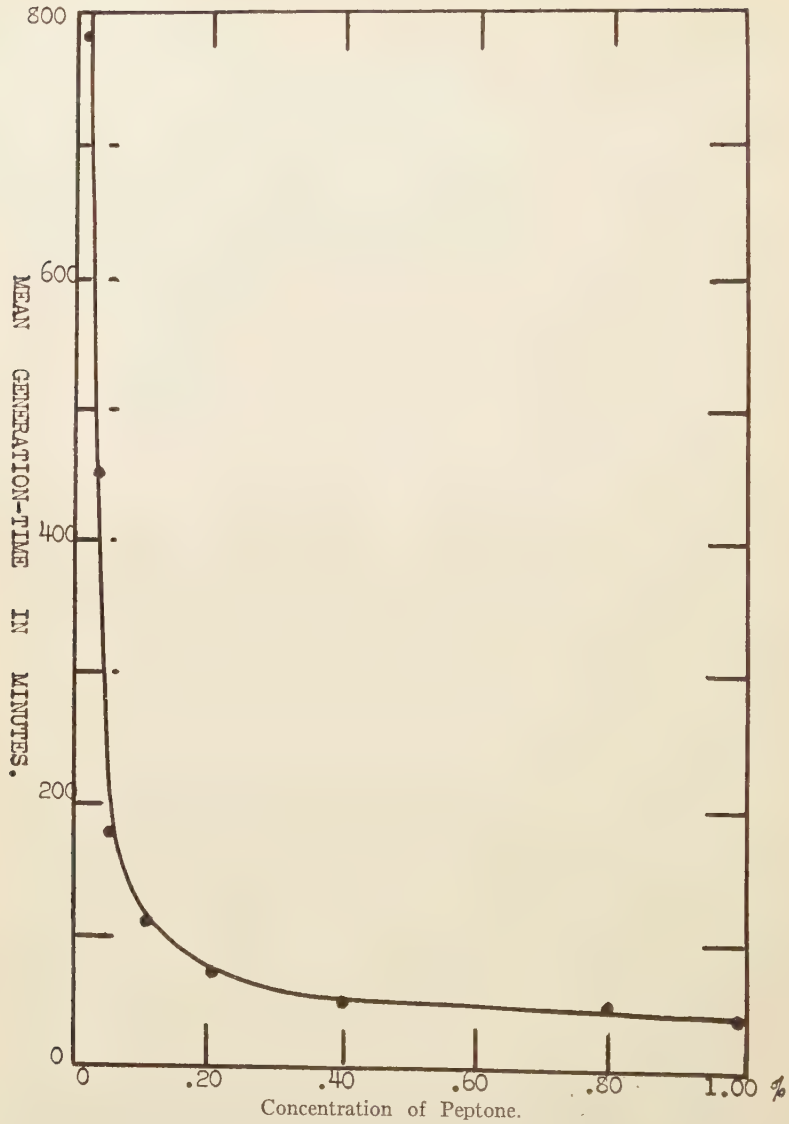

FIG. 7. INFLUENCE OF CONCENTRATION OF NUTRIENTS ON RATE OF GROWTH OF BACTERIA.

From "The Relation of the Concentration of Food Supply to the Generation-Times of Bacteria" by W. J. Penfold and D. Norris. Reproduced through the courtesy of the Journal of Hygiene. (1913, 12, 527).

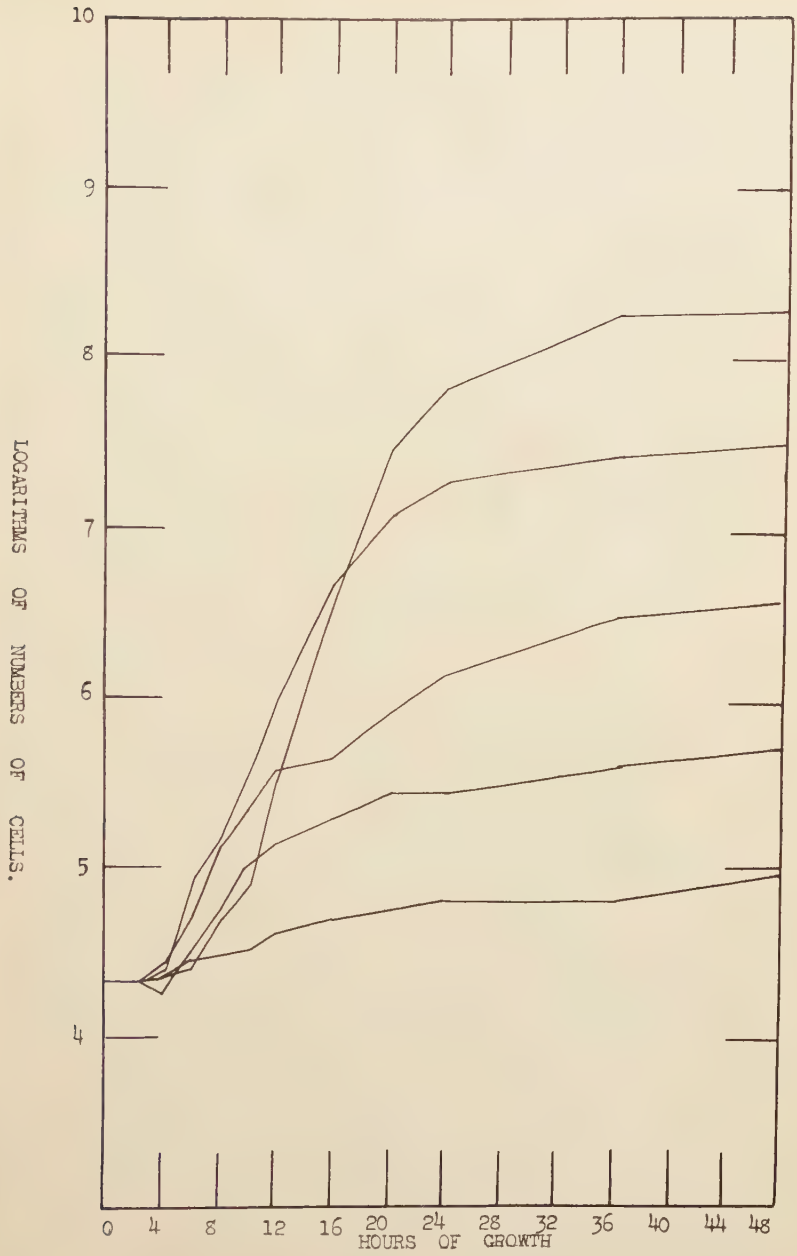

FIG. 8. INFLUENCE OF CONCENTRATION OF NUTRIENTS ON GROWTH CURVES OF YEAST.

of growth was roughly proportional to the concentration of the medium, as was the final yield.

Graham-Smith has contributed some valuable information on the relationship between the concentration of foodstuff and the growth of bacteria, though his observations are confined to the later stages of growth and are therefore not useful in determining the relative effects upon the lag and logarithmic phases. Figure 9 is copied from his paper, and shows curves for the number of cells in five cultures in media containing graded concentrations of meat extract. It will be noted that the maximum number attained is proportionately greater with increasing concentrations of nutrient, and also that growth is continued for a longer period with increasing amounts of extract. Further experiments showed that after growth had come to a standstill and the number of cells was decreasing, the addition of new meat extract in concentrated solution led to the development of a new cycle of growth, and that this could be repeated a number of times. By adding small quantities of extract in concentrated solution daily to the cultures, the death phase could be postponed for some time, the number of living cells remaining at a constant level. The addition of distilled water, diluting the medium, decreased the number of cells per unit volume but not the total number of cells in the flask.

It would appear, therefore, that the concentration of available foodstuff is a factor of prime importance in determining not only the rate of growth but also the final yield, the duration of the growth period, and possibly also the rate of death; and that the concentration of nutrients bears a reciprocal relation to the initial density of the population in determining the form of the growth curve, in conformity with Pearl's "law of growth" stated at the beginning of this chapter.

Some information is available on the influence of various other factors upon the rate of growth of bacteria. Salter found that the inhibitory effect of crystal violet on *B. coli* was more pronounced on young cells than old ones, and acted more in prolonging the lag phase than in reducing the rate during the logarithmic phase. Curran found that a large series of compounds added in small quantities tended to prolong the lag phase. Graham-Smith similarly found

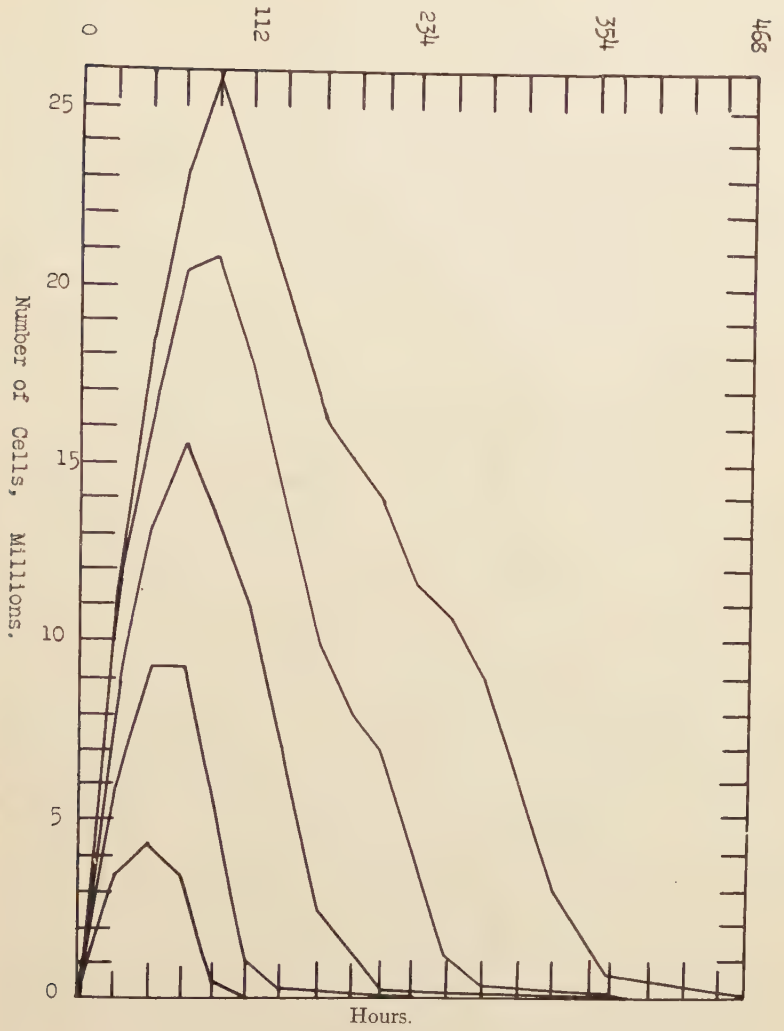

FIG. 9. INFLUENCE OF CONCENTRATION OF NUTRIENTS ON GROWTH CURVES OF BACTERIA.

From "The Behavior of Bacteria in Fluid Cultures as Indicated by Daily Estimates of the Numbers of Living Organisms" by C. S. Graham-Smith. Reproduced through the courtesy of the Journal of Hygiene. (1920, **19**, 131).

that the addition of acid and alkali to the medium tended to retard growth during the earlier phases, though subsequently growth proceeded at the normal rate. These results may be explained by the fact demonstrated by Sherman and Albus (1923) that "physiologically young" cells of bacteria are much more sensitive to various injurious agents than are older ones. Sherman, Holm, and Albus have shown that the accelerating effect of small quantities of neutral salts added to the medium is due to both a shortening of the lag period and an actual increase in rate during the growth phase.

In considering the growth curves of bacteria, two main problems, more or less interrelated, present themselves, viz: Why do the organisms stop growing, and why do they not start to grow at once with maximum rapidity when introduced into fresh medium? These have both been the subject of much theorizing.

Three explanations have been offered for the first problem: Growth ceases because the foodstuff has been exhausted and the organisms are starving; or because certain by-products of growth, either excretory materials or specific "autotoxines" are injurious to the organisms; or because actual physical crowding limits them.

A little thought will show that this last theory is quite untenable. When grown on the surface of agar, the organisms are more closely crowded together at all stages of growth than is ever the case in liquid media, and during the later stages are packed together as closely as they can be, yet with practically all aërobic bacteria the growth rate is higher on agar than in broth, and the final yield is higher than is the case with an equal volume of broth of the same nutrient value. Clark has shown that by growing yeast in a paper filter with the wort continually added, the yield was practically as high as that obtained when allowed to grow free in the wort, though the yeast cells were actually packed in a solid paste. I have similarly found that when yeasts are grown in a paper extraction thimble through which they could not pass, suspended in a large flask of dextrose broth, the total yield closely approached that in the control flask where the cells were free to diffuse through the medium; a similar experiment with colon bacilli contained in a collodion sack did not give a yield so closely approaching the expected amount, but still the actual density was much greater than when grown

free in the broth. Finally Rogers and Whittier have found in the case of *Streptococcus lactis* that if the effect of crowding is eliminated by repeatedly filtering off the organisms during growth and returning the filtrate to the parent culture, the period of multiplication and fermentation is not prolonged.

The idea that growth is limited by the accumulation of some toxic substance is the one that seems to be most generally accepted, though the evidence for it is far from being convincing. It is difficult to say who first suggested this theory, but Eijkman was one of the first to clearly formulate it. He grew the colon organism in gelatine for fourteen days at 37 degrees, divided the gelatine into two parts, one of which was heated to boiling, the other left unheated, and allowed both to solidify in culture dishes, after which they were inoculated on the surface with the same strain. No growth occurred in the unheated culture; a good growth occurred on the heated gelatine. Three possible explanations are offered: (1) That the medium was exhausted of nutrients which were set free again in the medium by heating; (2) that during growth antagonistic products of metabolism accumulated which were destroyed by heat; (3) that a diffusible thermolabile inhibitory substance was formed during growth.

The first possibility was excluded because after removing the bacteria from the unheated gelatine culture by filtration, it again yielded a good growth; therefore the nutrients were not exhausted. Herein lies a fallacy which has been repeated by almost all of the workers on this problem. It is assumed that growth should continue as long as any nutrient remains, taking no account of the amount necessary to merely sustain existence. The amount of food utilized by a cell during any given period of time will depend not only upon the amount of food present, but also on the number of cells present, and a culture which contains only sufficient food to maintain existence without growth for a population of say a million cells per c.c. will have enough to permit a very active growth of a population starting with say ten cells per c.c.; and similarly a medium might yet contain enough nutrient to support an active growth of a small number of cells, when with a very large number they would be actually starving and dying. That is, the occurrence or absence of

growth, or the relative rates of growth, depend not on the absolute concentration of nutrient, but on the amount of food available per cell. The occurrence of growth in a medium in which organisms have grown and from which they have been removed is therefore no proof that their cessation of growth in that medium was not due to a partial exhaustion of the foodstuff present.

Eijkman's filtration experiment indicated that if a toxic substance were active it would not pass a porcelain filter, even if the latter were paper thin. Further experiments showed that the growth inhibiting substance was destroyed by any temperature continued long enough to kill the organisms, and by volatile antiseptic substances —ether and ammonium sulphide—which could be evaporated from the medium after they had acted. Similar results were obtained with a number of other species of bacteria.

A number of further interesting experiments were made with the gelatine plate technique. If the "coli-gelatine" (gelatine in which coli had grown, resolidified in a plate) were inoculated with coli and covered with a layer of fresh gelatine, no growth occurred. If a fresh gelatine plate was streaked with coli and covered in part with fresh gelatine, in part with coli-gelatine, the former part grew, the latter did not. This was interpreted as indicating that a diffusible inhibiting substance passed out of the coli-gelatine into the fresh medium. Similar experiments were made, covering the coli-gelatine with paper dipped in agar; inoculation of the latter failed to give a growth unless the coli-gelatine had been heated. This experiment was made to prevent the possibility of organisms migrating from coli-gelatine to the upper layer of medium.

From these experiments Eijkman concludes that growth inhibition is due to a diffusible, very thermolabile substance formed by the bacteria themselves. Further experiments showed that it possessed a certain degree of specificity, inhibiting some closely related organisms but not other species. He failed to explain its failure to pass through porcelain filters in spite of the fact it would diffuse through agar and gelatine, and also found that it could not be separated from the bacteria by centrifuging.

It will be noted that in none of the above experiments was the hypothetical inhibiting substance separated from the living bacteria

save by a relatively thin layer of agar or gelatine, and in all cases the results can just as well be explained on the ground that no growth occurred because there were too many cells present for the amount of nutrient available. As Rolly pointed out, the apparent diffusion of the toxic substance into the fresh gelatine laid on the coli-gelatine might be explained by a diffusion of nutrients from the former to the latter.

Conradi and Kurpjuweit criticized Eijkman's technique on the ground that he used gelatine and agar as media, colloidal substances which might adsorb the toxic substance and so decrease its activity. They explained the failure of the substance to pass a porcelain filter as being due to this adsorption. They conducted experiments similar to those of Eijkman, allowing their organisms in broth which, after varying periods of incubation, was added to molten agar which was poured into culture dishes. They claimed that no growth occurred in these agar plates even though the living broth culture had been added to the agar, but this was not true, the number of cells introduced being so great that only microscopic colonies occurred, which were apparently overlooked by Conradi and Kurpjuweit, but were demonstrated in similar experiments by Manteufel and by Rolly. After the agar had solidified it was inoculated on the surface with the same or some other organism, and the presence or absence of growth determined. They concluded that all organisms produce more or less specific inhibitory substances which they called "autotoxines," which are formed in quantities parallel with the growth of the organisms themselves, reaching a maximum at twenty-four hours and not decreasing until after some days, comparable with phenol in potency, highly thermolabile, diffusible, and very easily adsorbed. The same criticism will apply to their experiments as to those of Eijkman, namely, that the inhibition of growth is due to an excess of living organisms within the medium. They claimed to have separated this autotoxine from the bacterial cells by dialysis. This could not be confirmed by Oebius or by Kauffman, and I also have failed to note any inhibition of growth in broth in which had been suspended a collodion sack containing a broth culture of the same bacillus.

Rahn, working with *B. fluorescens liquefaciens,* also concluded

that the inhibition of growth was due to a non-filterable thermolabile substance. Unlike Eijkman, however, he found that the organisms could be killed with ether (subsequently evaporated off) without destroying the inhibitory effect. A heated forty-eight-hour culture, re-inoculated, gave a good growth; a similar culture treated with ether gave no growth, but when heated later again allowed growth to occur; a tube of sterile broth treated with ether as above gave a good growth, showing that the ether treatment itself was not inhibitory; the same was true when a heated forty-eight-hour culture was treated with ether. Here is a clean-cut experiment which would seem to allow but one conclusion, namely that some substance inhibitory to growth had been formed, which was destroyed by heat but not by ether. The failure to pass a porcelain filter was explained by its adsorbability; small pieces of a filter placed in an ether-treated tube served to destroy the inhibiting effect. A number of other organisms were tried, but most of them could not be killed by the ether treatment; only three others, *B. lactis erythrogenes, Vibrio lactis,* and *Micrococcus grossus,* gave positive results. One might suspect that here we are dealing with a special case not generally applicable to bacteria at large, that perhaps the substance is pyocyanase or a related substance.

Chesney observed that curves of death of pneumococci in cultures appeared to follow the monomolecular law, as had previously been shown to be the case when bacteria are killed by disinfectants; and that when pneumococci are centrifuged out of an actively growing culture the remaining cells continue to grow actively, but if the culture be centrifuged after the period of maximum growth the remaining cells in the supernatant liquid show a prolonged lag and may even die for a time. Culture filtrates from twenty-four-hour cultures tended to inhibit the growth of actively growing pneumococci inoculated into them, the inhibitory action being lost if the filtrates were held for a time at incubator temperature. Actively growing pneumococci exposed to the action of a twenty-four-hour culture filtrate at low temperature showed a longer lag than controls. Filtrates of four-day-old cultures failed to inhibit. He concludes that the inhibition of growth is due to some unstable toxic product of growth which injures the cells. M'Leod and Govenlock also be-

lieved that the limitation of growth of pneumococci was due to the accumulation of a thermolabile toxic substance which they named "bacterocidin," but in later papers M'Leod and Gordon showed that this substance was hydrogen peroxide and that it was produced by several other organisms, notably the streptococci. This was confirmed by Avery and Morgan, and Morgan and Avery concluded that the limitation of growth in pneumococcus cultures is due to three factors, the accumulation of acid, the production of peroxide, and the exhaustion of the nutrients. These findings would indicate that here again we are dealing with a special case not generally applicable to all bacteria.

Hajos grew various members of the colon-typhoid group in broth until a maximum growth was attained, centrifuged out the bacteria, re-inoculated, and incubated again, and repeated this process until no further growth occurred. This exhausted medium was distinctly inhibitory to bacteria; a few drops added to 10 c.c. of fresh broth was sufficient to produce a definite inhibition of growth. The toxic substance was merely inhibitory, failing to kill bacteria suspended in it, was not specific, was not destroyed by heating to boiling, and passed through a Bechold ultrafilter. This substance is quite different from those previously described. Hajos considers it to be a product of metabolism rather than a specific "autotoxine." This substance can, however, hardly be of great importance in the cessation of growth in normal cultures, since it could only be obtained in quantities sufficient for demonstration after the medium had been completely exhausted by repeated subcultures.

Curran, observing that growth occurred in media in which bacteria had previously reached a maximum growth, and from which they had been removed by heating or by filtration, also concluded that the cessation of growth is due to a thermolabile substance readily adsorbed by bacterial filters. To test the latter theory more carefully, he performed the following experiment. A three-day old culture of *B. coli* was passed through a filter, the first and last 50 c.c. portions of the 200 c.c. culture being retained. These two portions were tested separately for their ability to support growth; the culture in the last portion grew more slowly, only half as many organisms being present after ten hours as in the first portion, though

both cultures were equally and simultaneously seeded. He concludes, therefore, that there is a toxic substance which is adsorbed by the filter, but that after a certain period of filtration the filter becomes saturated and allows the substance to pass through. This experiment, like the filtration experiment of Chesney, is very suggestive, but cannot be considered conclusive until it has been repeated a sufficient number of times to show that it is universally true.

With the relatively recent discovery of the bacteriophage, the question has naturally arisen whether or not these supposed autotoxic substances formed in cultures of bacteria might not be the d'Herelle principle. Hajos says that the inhibitory products of metabolism which he obtained from exhausted cultures have nothing in common with the bacteriophage. Otto and Munter, apparently accepting as valid Conradi and Kurpjuweit's autotoxine, showed that the latter was not neutralized by antilysin. Kauffman, denying the existence of the autotoxine, naturally concludes that it is not identical with the lytic substance of d'Herelle.

While many of the experiments reported above are very suggestive of the occurrence of toxic products which tend to limit growth, and one cannot, therefore, categorically deny their existence, there is nowhere any clearcut evidence that such substances are universally formed, sufficient to warrant the prevalent acceptance of the idea. The observations of Penfold and Norris, Salter, and Graham-Smith with bacteria, of Brown, Zikes and Montank with yeasts, that the rate of growth and the final yield are proportional to the concentration of nutrient in the medium, seem to offer a serious obstacle to the toxic theory, for it seems more likely that toxic products of growth would accumulate more rapidly and thus inhibit growth sooner with a medium of higher nutrient value, rather than the reverse. The crucial experiments of Graham-Smith, in which he showed that a culture of staphylococci could be revived at any time after the maximum growth period and caused to grow again by simply adding some more meat extract in concentrated form, and that the death phase could be postponed indefinitely by small daily additions of meat extract, would seem sufficient to completely upset the toxic theory, unless one can believe that meat extract is an antidote to the autotoxine!

If the cessation of growth is due to a partial exhaustion of the medium, repeated subcultures on the same medium, with removal of the organisms after each culture, should behave as cultures in media decreasing in concentration of nutrients, and one should obtain a series of growth curves similar to those in Figure 8. While there is much evidence in the literature that such repeated subcultures do yield steadily diminishing amounts of bacteria, there is very little quantitative data available. Rahn measured the rate of growth in successive cultures of *B. fluorescens* in parallel series heated to 100 degrees and 68 degrees, but the various cultures were not equally seeded, and growth was not continued to the maximum in each case, so that the figures are not directly comparable. The 68 degree series gave the following mean generation times for the first twenty-four hours of growth in five successive crops: 98, 96, 108, 108, and 103 minutes, certainly not enough difference to be significant. In these cultures, of course, the dead cells remained in the medium, and it is probable that on heating some nutrient was liberated from them and returned to the medium, and that during growth further food was made available by the action of enzymes secreted by the living cells upon the dead ones. The amount of nutrient thus made available, however, must be small. Similar studies with filtered or centrifuged cultures would be more significant.

Curran made an experiment similar to the above, using *B. coli,* the medium being autoclaved and reseeded every four days. The first culture yielded 500,000,000 cells per c.c.; the four subsequent crops yielded about 100,000,000 each time. He also measured the rate of growth hour by hour in a culture which had been heated, with a control in fresh broth, and another culture from which the bacteria had been removed by filtration with a similar control. The results of the two experiments were very similar; in neither case was there any difference in the duration of the lag phase between the used broth and the fresh medium, while in both cases there was a decrease in the rate of growth during the late logarithmic phase in the used broth.

The apparent contradiction in the findings of various authors with regard to the limitation of growth in cultures may be explained

if it be agreed that different factors may limit growth in different cases, i.e., that every culture is a special case like the pneumococci, and that there is no generally applicable law. This is probably true. Thus Brown found that in ordinary flask cultures of yeast, oxygen was definitely the limiting factor; if the cultures were well aërated, food seemed to be the limiting factor, the yield being greater in wort of higher specific gravity. Clark believed that in aërated yeast cultures the accumulation of alcohol was the limiting factor, but the work of Balls and Brown would indicate that under such circumstances the concentration of dextrose determines the rate of growth. Rogers and Whittier have found that in cultures of *Streptococcus lactis* the accumulation of acid is normally the limiting factor; if this be removed by keeping the hydrogen ion concentration constant, then growth proceeds to a higher level which is determined by the oxygen concentration, a still higher level being reached if the culture is aërated. Probably similar different limiting factors will be discovered in each case, and if they are removed the final factor will be found to be the concentration of foodstuff. At least there is no sound basis for believing in the production of specific autotoxines.

Many theories have been proposed to explain the period of latency at the beginning of the growth curve. Chesney has well divided these into two groups, those which seek the origin of lag in the medium, and those which find the cause in the inoculated cells.

Rahn found that cultures seeded into a medium in which the same organisms had previously grown and had been killed by heat, had little or no lag, although there was a definite latent period in control cultures on new medium. There was a definite lag, however, when organisms were recultivated in a medium from which the preceding crop had been removed by filtration. He concluded that the maximum growth rate could only be attained at a definite concentration of some heat stable, non-filterable substance formed by the bacteria themselves.

This idea that the lag is due to the lack of some substance secreted by the bacteria has also been held by various other authors, but more particularly by Robertson, who has referred especially to a similar phenomenon in the growth of protozoa. He lays especial

stress upon the fact that the lag period is shortened by increasing the size of the seeding, and assumes that this is due to a mutual stimulation of the cells by each other. Since the cells are separate, this can only be accomplished by their shedding something into the medium, and this, Robertson states, is a catalytic agent liberated during the process of cell division. This mutual stimulation of the cells Robertson refers to as the "allelocatalytic effect."

But Cutler and Crump, and Greenleaf, failed to observe any allelocatalytic effect with protozoa, and Peskett had similar negative results with yeasts. Curran failed to observe any shortening of lag when bacteria were seeded into broth separated from an actively growing culture by a collodion membrane; therefore if there is an extracellular catalyst it is not diffusible. But, as was demonstrated by Chesney, there is no lag if the organisms are taken from a culture in the maximum growth phase, and this was found to be true regardless of the size of the seeding by Sherman and Albus (1924b), growth continuing at a maximum rate if the inoculum was but one cell per c.c. It would seem, therefore, that if lag is due to the lack of some extracellular catalyst, this catalyst is required only by cells which had passed the logarithmic growth phase in the preceding culture. As will be pointed out later, the effect of the size of the seeding can be readily explained on the basis of variability in the inoculated cells with regard to their ability to grow; the larger the number introduced, the greater the chance for obtaining rapidly growing individuals.

Since actively growing cells show no lag and cells taken from a culture which has stopped growing do, Chesney rightly concluded that the cause of the lag is to be found not in the medium, but in the cells, and seeks this cause in an injury which the cells have sustained in the preceding medium, and from which they must recover before they can start to grow. He believes the cause of this injury to be the toxic substance above mentioned. But proof of the general existence of such a toxic substance has so far not been furnished. However, this theory will still hold if we believe that the cessation of growth is due to a lack of nutrients, and that the cells have been injured by starvation. It would seem, at least, that whatever causes a slowing and final cessation of growth

in the culture also somehow changes the cells so that they are not immediately able to respond to fresh medium by growth; whether this change is of the nature of an injury, and just what is the cause, is not clear.

The latter view is practically that of Buchanan, namely that the cells of a culture which has ceased growing are changed into "resting" cells physiologically analogous to spores or seeds, and that when introduced into fresh media these must "germinate" before they can grow. The lag phase is the time required for this germination. Buchanan's theory postulates, therefore, a functional difference between growing cells and resting cells, and it is interesting to note that such physiological differences have been demonstrated by Sherman and Albus (1923), and to anticipate somewhat by stating that I have found corresponding morphological differences. Sherman and Albus (1924a) also explained lag on this basis, observing that the increased sensitivity of "physiologically young" cells could be demonstrated before the maximum growth rate had developed. It is also interesting to note in this connection that Durbin explained the latent period in the growth curves of regenerating tadpoles' tales as being due in part at least to the time required for the adult cells of the stump to transform to embryonic cells capable of growing.

Buchanan first divided the lag phase into two parts, an initial dormant period and the true lag phase or period of accelerating growth. The former may be quite prolonged, as has been observed by Burke and his co-workers, who believe that such prolonged periods of dormancy, especially observed with spores, have nothing to do with the ordinary lag phase. But I believe that the difference is only one of degree, and that the short period of dormancy commonly observed is the same sort of a phenomenon. During this period of dormancy some of the cells may actually die, and as will be seen later, they may also show morphological changes of the same sort as they did in the parent culture. This period of initial dormancy, then, is not entirely occupied by a transformation, either physiological or morphological, to a new type of growing cell. It may perhaps best be explained in the terms of Penfold, as being due to an "inertia", i.e., to the bacteria continuing to react for a time in the new medium to a stimulus received in the old.

Penfold has suggested another theory that deserves mention, namely that "the inoculum consists of organisms having individually different powers of growth, and that during the lag the selection of a quick growing strain occurs in response to some selecting agent in the peptone". This explains the gradual acceleration of growth in the true lag phase. It will be seen from later chapters that such individual variations in power of growth occur, and that a selection of a rapidly growing strain may take place.

CHAPTER III

ON TECHNIQUE

In the work here reported, two things were desired: To obtain as accurate as possible a measurement of the rate of growth of the cultures studied, and also to obtain, as far as possible clear microscopic preparations of the cells, from which reasonably accurate measurements of their size and form could be made, since the purpose of the work has been to study morphologic variations of bacteria quantitatively, and to correlate these variations as far as possible with the rate of growth. For various reasons it was necessary to use solid media for the growth of the organisms. First, because it is difficult to obtain clear microscopic preparations from liquid media, because of the presence of peptone or other material which obscures the picture; and second, because it is necessary to obtain considerable numbers of cells for the purpose of microscopic observation during the early hours of growth when they are relatively few in number in the medium. If liquid media were used, it would be necessary to centrifuge, wash, and recentrifuge, in order to clear the organisms of the accompanying debris of the medium, and to sufficiently concentrate them. In addition to being time consuming, it was thought likely that this process would be apt to lead to some artificial variations in the morphology of the cells.

Broth cultures were, however, used for certain studies of *B. megatherium* described in Chapter IV (Cultures II-VI). Since this organism grows very slowly in liquid media because it grows only at the bottom and therefore lacks oxygen, some method had to be used for providing aëration. In Cultures II and III this was accomplished by inoculating 1,500 c.c. quantities of broth, and then distributing this in 60 c.c. amounts into Kolle flasks, so that the broth was disposed in a thin layer with a large surface exposed to the air. A flask was removed for sampling after each hour, assuming that the conditions of growth were identical in all of the flasks. In Cultures IV, V, and VI, 1,500 c.c. flasks of broth were also inocu-

lated, but aëration was accomplished by thoroughly shaking the flasks every half hour during growth. Samples were removed from the flasks with sterile pipettes.

The difficulties met with in broth cultures can be obviated by using solid media, agar slants, the cells being sufficiently concentrated and obtained practically free of products of the medium itself by simply scraping them off with a wire loop. The use of agar, however, introduced certain other difficulties. In an ordinary agar slant the medium varies in depth from the bottom to the top, and the conditions of growth are therefore not uniform throughout the tube. As a matter of fact, growth is heavier and probably also more rapid in the lower part of the tube. In order to obtain a representative sample from agar slant cultures, then, it is necessary to suspend the entire growth in water and remove a sample from this suspension for examination. This necessitates using at least one agar slant culture for each observation, and therefore the preparation of a large series of agar slant cultures as uniform as possible in size of seeding and conditions of growth for the entire period of observation. This was approximately accomplished by using a large series of measured test tubes of practically uniform diameter. These were obtained by first boring two holes into a piece of sheet brass, differing in diameter by about one-half millimeter. Test tubes as they came in the commercial packages were then selected by means of this piece of brass, only those tubes being chosen which would pass through the larger opening but not through the smaller one, the variation in diameter of the tubes thus being practically negligible. These tubes were then filled, each with 5 c.c. of agar carefully measured, and were also sloped, as far as possible, at the same angle. The tubes, therefore, were probably sufficiently uniform for the purposes, the difficulty being in obtaining uniform inoculation.

Seeding was accomplished by preparing a suspension in sterile water, or by using a peptone or broth culture, and one loopful of such a suspension was spread as uniformly as possible over the surface of each slant. Undoubtedly the loopfuls varied somewhat in size, and more particularly it was difficult to maintain uniformity of distribution of the seeding over the surface of the agar slant. At any rate there was a certain amount of unavoidable variation in

the results, which could only be explained as being due to a lack of uniformity in the various tubes. This was most noticeable in the early hours of growth.

In all of the work standard beef extract agar and bouillon were used as culture media, adjusted to pH 7.

The cell counts and microscopic preparations for purposes of measurement were made from the growth on the above mentioned agar slant cultures suspended in sterile water. For the later stages of growth, where the number of cells was large, generally one or two tubes were removed from the incubator and the growth washed off with water and used for preparations for counting and measuring. Samples for counting were immediately killed by adding formalin. In the early stages of growth, however, the number of cells is so small that it is necessary to remove a number of tubes in order to obtain a sufficient number of cells for reasonably accurate counting, and particularly to obtain a sufficient number of cells for microscopic preparation and measurement.

The procedure followed was to remove a number of tubes and add to the first 2 or 5 c.c. of water. The bacteria were then scraped off into this water, which was poured into a second tube, and this process repeated from tube to tube until enough bacteria had been removed to give a faint but perceptible turbidity to the liquid. The number of tubes used and the amount of water used was varied according to the stage of growth and the number of bacteria actually present in the cultures, the final calculations being made so that the number per c.c. is expressed as though the growth from one tube had been suspended in 2 or 5 c.c. of water, as the case may be.

Of the various methods which offered themselves for obtaining the cell counts necessary for plotting growth curves, one of two microscopic methods was used in each case. There were various reasons for this, the most important being that with some of the organisms studied the organisms tend to clump or to form long chains, and the degree of clumping and chain formation varies with the age of the culture. Plate cultures, therefore, would introduce a serious source of error, which can be more or less obviated by the microscopic method. With *Bacillus megatherium,* which is relatively large, counting was done by the use of the Helber counting chamber.

With this organism all of the cell counts were made with the same counting chamber and cover glass, so that whatever absolute error there may have been, there is no error in the course of the growth curves due to this technique. Wilson has concluded that when the number of cells is lower than fifty million per c.c. the error of the counting chamber method becomes too great to permit accurate results, but it is evident that this depends entirely on the number of counts made and averaged for the final results, i.e., on the total number of cells counted regardless of their concentration. With the agar slant cultures the cells were actually concentrated during the early hours of growth until the liquid showed some turbidity, which generally indicates about a million cells per c.c., so that the count is the average of a number of tubes. This method involves a certain amount of error in that not all of the cells are washed off in the collecting process. With the broth cultures no such concentration was possible, and the counts during the early hours of growth had to be made by patiently going over one preparation after another until a sufficient number of cells was counted; in general a thousand cells were chosen as the limit, but in the very early hours of the lightly seeded broth cultures this was quite impractical, and the counts here are admittedly not of a high degree of accuracy.

With the other organisms, however, because of their small size and their tendency to remain floating in the suspension, the counting chamber method was too difficult and too prone to error to be useful. For all of these other organisms, therefore, I have used a direct microscopic counting method devised by myself, but in principle essentially the same as that introduced by Breed and Brew for counting bacteria in milk. The suspensions of organisms were mixed with an equal volume of a 2 per cent solution of Congo red, and .01 c.c. of this mixture was spread as uniformly as possible over an area of four square centimeters on clean glass slides. During later phases of growth, where the number of organisms is larger, eight square centimeters were sometimes used. The Congo red preparations, after drying, were then placed a moment in a solution of 1 per cent hydrochloric acid in alcohol, which serves to fix the film and also to change the Congo red to a deep blue. The counting of the organisms is then accomplished by counting the number

of cells in a number of representative fields on the slide, and computing the area of each field, and thus obtaining the number in the total preparation. This method presents certain difficulties. The films do not dry uniformly; that is, they are much thinner at the edge than at the center, and the cells are correspondingly more numerous in the center than at the periphery. It is necessary, therefore, to count a number of fields chosen at regular intervals along a line extending from one edge through the center to the opposite edge of the slide. In general, twenty fields were chosen, ten along each of two lines at right angles to each other, the spacing of the fields being easily accomplished by means of a mechanical stage. There is also some difficulty in accurately discharging .01 c.c. pipettes onto a slide, and leaving behind on the tip of the pipette the same quantity each time. It is therefore very desirable to prepare a number of slides, in most of the work here reported—five. The total number of cells counted in most of the cases, therefore, was the number contained in one hundred microscopic fields; that is twenty fields from each of five different preparations. This is tedious but necessary to obtain uniform results. Where the number of cells is small, as during the early stages of growth, frequently many more fields had to be counted to obtain a count of accuracy comparable to that during the later stages of growth.

Since practically all of the cell counts were made by direct microscopic methods, death of the cells is not recorded in the curves until the dead cells have completely autolyzed. Wilson has pointed out that some cells are dying at practically all stages of growth, but during the growth cycle proper the number of these is small, and the error is practically negligible. In the death phase, however, the microscopic counts do not give a true measure of the rate of death, since of course the curve does not begin to decline until some time after the cells have actually begun to die; and further, as will be shown in Chapter IX, because the rate of autolysis is not dependent upon the death rate. The death phase therefore began much earlier and the rate was probably much higher than is indicated in the curves given, particularly with the cholera vibrio as described in Chapter VIII. No particular attention has been paid to the death phase, however, save in studies of the involution forms of the colon

bacillus described in Chapter IX, and here the death curves were determined by plate counts.

The measurements of morphologic characters, size, and form of the cells, were accomplished in various ways. With *Bacillus megatherium,* because of its large size, these measurements were made fairly easily. Microscopic preparations were projected, using a 1.5 mm. apochromatic objective and a 12× periplan eye piece, with an arc lamp as illuminant, onto a screen at such a distance that the magnification was approximately 3000×. The individual cells were then easily measured simply by spreading dividers over the projected image and holding these dividers to a ruled scale. In some cases measurements have also been made by the use of the Abbe camera lucida, simply spreading the dividers along the table over the apparent image of the cells. For the smaller organisms, however, it is necessary to obtain a higher magnification, in order to have reasonably accurate results; and since projection at magnifications beyond 3000× is impractical, because of the difficulty in obtaining sufficient light, the following procedure was adopted:

Photomicrographs were prepared of numerous fields, using the above named lenses, the magnifications varying from 2000× to 3000×, according to the bellows draw, and the photographic negatives were again projected at a magnification of seven to ten times. All photographs were made on panchromatic film, using a Wratten red filter, which gave the maximum contrast. The projects images of the cells were then either measured directly, or more frequently they were traced onto individual cards and the drawings on these cards were then measured for area by use of a planimeter, and for length by means of dividers. This last procedure may be considered as involving considerable error, because of the extreme magnification, the final magnification being about 20,000×. Naturally with such magnifications the edge of the image is far from being sharp, there being a zone of fuzziness corresponding in dimensions to the limits of resolution of the microscope, that is, to about 0.15μ in terms of the original microscopic preparation. This error is, however, a compensating one, for in trying to place the dividers or trying to trace with the pencil as far as possible through the center of this zone of unsharpness, the chances are as great for going inside the middle

of this zone as outside of it; and where relatively large numbers of measurements are made, this error tends to cancel itself out. While on theoretical grounds none of these methods of measurement are as accurate as could have been obtained, for instance, by the use of a filar micrometer the large number of measurements made serves to give at least a reasonably close approximation to the true measurement, and because of the much greater length of time required, it would have been practically impossible to make all these measurements by use of a micrometer.

In the case of the colon bacillus (Chapter V) and the cholera vibro (Chapter VIII) I have attempted to illustrate the *average* size and form of the cells by means of composite photographs. These were prepared from the tracings of the cells on cards as described in the paragraph above. The proper exposure for one card was determined, and then all of the cards were photographed on one plate, each receiving a proportional share of the entire exposure. The cards were all so placed that the center of each cell came at the same point on the plate, and that the long axes of the cells coincided as nearly as possible. If the cells were curved, the cards were so placed that the major curvature always came on the same side.

Nearly all of the measurements have been made from slides prepared as above described, using the negative staining method of Benians, where the organisms are surrounded by a field of dark blue Congo red. This procedure was chosen because it seems to give the least amount of shrinkage of any of the methods used for making permanent microscopic preparations of bacteria. The image is not that of the bacterium itself as it appears after it has been dried and fixed, but of the space which was occupied by the cell before it underwent the shrinkage which occurs during drying and fixing. This fact, previously stated by Eisenberg, can be easily demonstrated, for with the large organisms, at least, one can see the shrunken cell within the space which is not occupied by the dye, there being a considerable zone between the cell and the edge of the dye. In a preliminary series of measurements of a preparation of *Bacillus megatherium,* measuring first the living cells suspended in water, then the clear spaces in the Congo red preparation, and finally a dried fixed slide stained with methylene blue, it was found that the measure-

ments of the Congo red preparation were practically identical with those of the living cells, whereas the methylene blue preparation gave dimensions about one-third less. With the Congo red method, therefore, the image of the cell is larger than with other staining methods, and the measurements are correspondingly more accurate.

Other negative staining methods could be used, as India ink or nigrosin; but India ink is more granular and tends to give a less sharp image, and at least some samples of nigrosin tend to become precipitated by certain substances associated with the bacteria, and similarly give a granular background with an unsharp image. The negative staining method also appears to give a much sharper image than is the case with slides stained in the ordinary way.

This negative staining procedure with Congo red gives such clear preparations for certain types of morphologic investigation that a word or two further concerning it will not be out of place. A perfect image of the cell is only obtained when the film of stain comes up to the very edge of the cell but does not extend over it. It is therefore not useful with organisms which accumulate any appreciable amount of slime about them, and only those parts of the film are to be used where the layer of stain is not so thick as to extend over the top of the cell. This latter point may be readily determined by examination of the preparation; if one observes the cells in a thick portion it will be found that they are relatively slender and not clear white, but as one proceeds to the thinner parts it will be found that they become larger, and that one soon arrives at a place where no decrease in the intensity of the blue is accompanied by an increase in the size and brilliancy of the image of the cells, indicating that the film is now so thin that it has not extended over the top of the cells. The Congo red is precipitated by peptone, and with other liquid media artifacts are produced by the precipitation of substances dissolved in the medium, so that the method is not satisfactory with organisms from liquid cultures unless they have first been centrifuged out and washed with water. For this reason cell measurements for the broth cultures described in Chapter IV have been made from preparations stained with methylene blue.

While in young actively growing cultures none of the cells show any portion stained with the Congo red, the entire cell appearing as

a colorless space in the film, this is not true in older cultures. Eisenberg has noted that with the Gram-negative bacteria there frequently appears a colored spot in the middle of the cell, which he interprets as being due to plasmolysis, the shrinkage of the protoplasm to the poles of the cell leaving a concavity in the center which becomes filled with dye solution; such colored portions were not observed with Gram-positive organisms which could not be readily plasmolyzed. I have also observed this, but in addition have noted that in *B. megatherium* blue stained granules appeared in the cell during the period of spore formation, corresponding in size and position with the sporogenous granules which could be demonstrated in preparations stained with methylene blue, and also that stained granules appeared in the preparations of the diphtheroid organism described in Chapter VI, corresponding with the volutin granules demonstrated by Ponder's stain. It would appear, therefore, that such structures are stained by the Congo red with the technique employed. It is hard to understand why such intensely basophilic granules should stain with an acid dye like Congo red, while the remainder of the protoplasm remains unstained. Ernst has also observed that the granules which bear his name are stained by Collargol (which, according to Eisenberg, behaves like Congo red), free granules contained within vacuoles in mold mycelium becoming stained a dark brown. There is, then, no question but that these colloidal negatively charged dyestuffs can penetrate within the cells.

With increasing age of the cultures it is also found that some of the cells become uniformly stained throughout by the Congo red. Because these stained cells become increasingly more numerous with increasing age of the culture, I have concluded that they are dead cells. But cultures killed by formalin or other fixative solutions do not become stainable by the Congo red, and cultures killed by heating to boiling show only a faint staining after some time. Cultures killed by heating to 60 degrees, however, show all of the cells distinctly stained after standing several hours following the heating. It would appear, therefore, that the mere death of the cells does not cause them to become stainable, but that the change in their reaction towards Congo red is due to some process which occurs after death, i. e., to beginning autolysis.

Various other staining methods which will differentiate living from dead cells have been described. As is well known, Gram-positive bacteria become Gram-negative in old cultures, and R. and W. Albert have shown that the loss of the Gram-staining property in yeasts is correlated with the degree of autolysis of the dead cells. Fraser studied the action of various dyes on living and dead yeast cells, and found that these could be differentiated readily by methylene blue; his procedure was to suspend the unfixed cells in the dye solution. This method was used by Fulmer and Buchanan to measure the death rate of yeasts acted upon by disinfectants; they pointed out that the ability of the cells to absorb stains occurs *after* the cells have lost the ability to grow when transferred to a new medium. Burke has shown that dead spores may be differentiated from living ones by staining with carbol fuchsin, and Koser and Mills also found that the ability of the spores to absorb the dye was acquired later than the period when they lost the power to germinate. Seiffert found that when suspended in Congo red dead cells of bacteria absorbed the dye, living ones not. But the differentiation by this technique is not so sharp as with the procedure I have used.

It has generally been assumed that the staining of the dead cells is due to increased permeability of the membrane to the dyestuff, and this view would seem to be supported by the observation of Fraser that dead yeast cells stain much more slowly with the less diffusible Congo red than with methylene blue. With Fraser's technique, however, it is found that with neutral red the dead yeast cells become stained a uniform deep red, and that in the living cells the volutin granules and vacuoles are also stained. The cell wall of the living cells must therefore be permeable to the stain, and the failure of the protoplasm to become stained must be due to a difference in its adsorption properties from those of the dead cells. As I have pointed out above, granules are also stainable in the living cells of bacteria when Congo red is used, so the membrane must also be permeable to this dyestuff.

The fact that dead, partially autolysed cells may be detected by the use of Congo red gives us a valuable procedure for measuring the rate of death and autolysis of bacteria which has been used in con-

nection with a study of morphologic variations during the death phase described in Chapter IX.

The statistical part of the work was greatly facilitated by the use of an adding machine, measurements being recorded on that instrument as they were observed, so that the total and consequently the average were immediately available. In the earlier work with *B. megatherium* recorded in Chapter IV, 300 cells were measured from each sample except during the period of great variability when 500 were measured (1,000 in the case of the five and one-half-hour sample from Culture I); 300 cells were also measured from each sample in the case of the diphtheroid organism described in Chapter VI; but it was apparent that nothing was gained in the way of smoother curves by using such large samples, and the number of cells measured was reduced to 250 in the case of the colon bacillus (Chapter V), and to 200 in the succeeding studies. Cell measurements were made to the nearest millimeter of the projected image, and this unit was chosen in most cases for the class intervals of the frequency curves; the class intervals are therefore smaller with the higher magnifications. The various statistical constants have been computed by standard methods save the modes and medians. Modes have been determined simply by inspection of the smoothed frequency curves, medians from the (unsmoothed) cumulative percentile curves. The frequency curves presented in the succeeding chapters have all been smothed twice by the method of three-point averages. None of the other curves have been smoothed save those shown in Figure 25 and Figure 27, which have been fitted by simple inspection.

CHAPTER IV

THE SIZE OF THE CELLS OF BACILLUS MEGATHERIUM

While it has been well known for a long time that in old cultures the form of the cells is different from that which they exhibit in younger cultures, it has been only relatively recently demonstrated that in very young cultures, that is during the active growth period, there is also a difference in the cells from those in the standard twenty-four-hour culture. This was demonstrated by Clark and Ruehl, who have shown that during the early hours of growth there occurs a marked increase in size of the cells with most species of bacteria. They observed this in *Bacillus typhosus,* B. *paratyphosus, B. coli, B. influenzæ, B. pyocyaneus, B. pertussis, B. anthracis B. subtilis, B. vulgatus, B. avisepticus,* and the cholera vibrio. The same thing was observed with *Streptococcus pyogenes,* but not with the glanders organism, the diphtheria bacillus, Hoffman's bacillus or *Bacillus xerosis,* the last four showing a decrease rather than an increase in the size of the cells. They note that the change in the size of the cells is in some instances so great "as to render the organism unrecognizable when viewed by the ordinary standards of the twenty-four-hour culture." They found that with a large number of the cultures studied there was some increase in size apparent after two hours incubation, the maximum size being reached on the average from four to six hours after inoculation. They state that "the correlation in time of the occurrence of the long, coarse forms, with the period when the maximum growth of the culture is taking place, is obvious. That the cross section of the culture with its very short average generation time should show a majority of forms dividing and nearly ready for further division, is also obvious." This latter statement, however, cannot be accepted. If the increase in size is to be explained merely by the growth required for the cells to attain a sufficient length for division to occur, that is twice their original size according to our previous conceptions of the process, then the maximum size attained should never be more than one and one-half

times the normal size, since in any given sample there will be just as many cells recently divided as cells about to divide. As a matter of fact, in their own observations, in a good many cases the maximum size attained was considerably more than this amount, and according to my own studies may be as much as six times that of the resting cells. Moreover, if this is the explanation, it should apply equally well to all bacteria, including the diphtheria group, which, as they observed, decreased, rather than increased during the maximum growth period. As will be seen later, there is much evidence that this change in the size of the cells is correlated with other changes in their morphological characters, and also in their physiology, and that the real significance of the phenomenon is that the actively growing cells are fundamentally different from the resting cells, and that we have in a *young* culture a special morphological type which differs just as much from the standard type that we know from observations of twenty-four-hour cultures, as do the so-called involution forms which we find in old cultures.

I have studied this phenomenon of increase in size in some detail, particularly with *Bacillus megatherium*, chosen because it is the largest bacterium that can be easily grown in artificial culture media, such a large organism making measurements of size relatively easy and more accurate than is the case with smaller bacteria. The strain used was one isolated from soil, which corresponds with the published descriptions of *Bacillus megatherium* in every respect save that it is non-motile. This quality of motility in the megatherium group seems to be one which is extremely variable. In addition to its large size this organism is also characterized by the occurrence of numerous intracellular granules, rather more refractile than the protoplasm which contains them. They do not give the staining reactions of either volutin or fat; in fact they do not stain with any of the dyes. But in the fact that they disappear during active growth and reappear when growth slows up they behave like volutin and possibly serve a similar function.

The general character of the morphologic variations exhibited by this organism may be seen in Plate 1, which shows camera lucida drawings of living cells removed from a culture at various stages of growth. There was no appreciable variation in the cells in the first

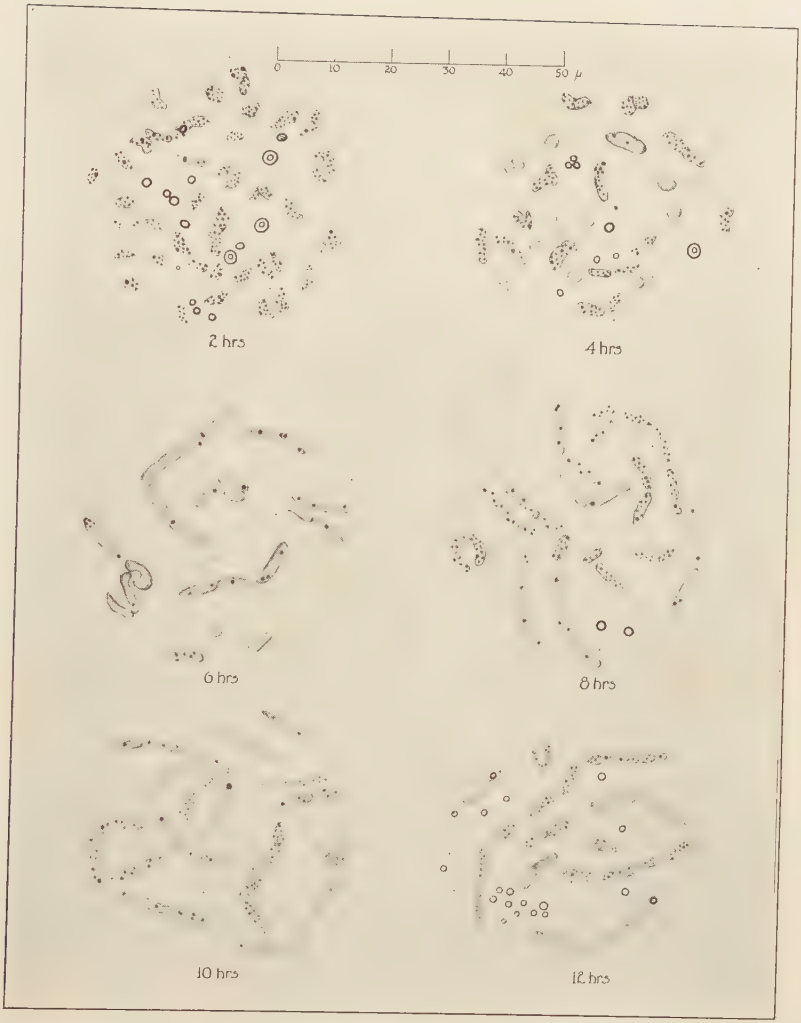

PLATE I. MORPHOLOGIC VARIATIONS OF *B. megatherium* AT DIFFERENT STAGES OF GROWTH.

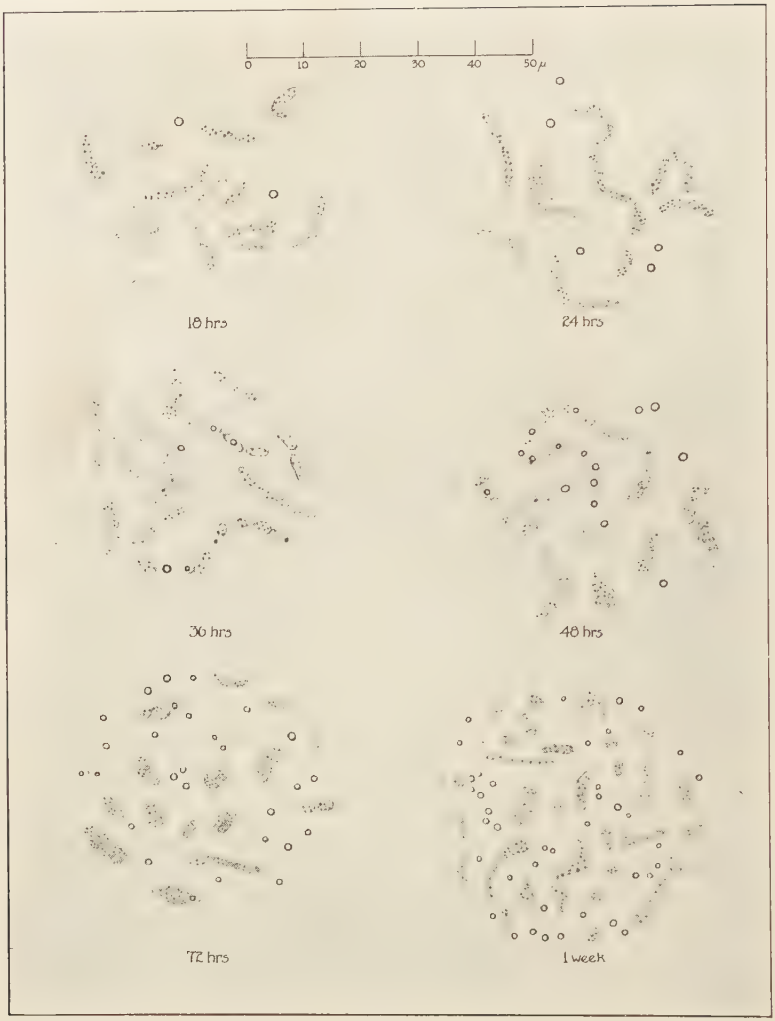

PLATE I (continued) MORPHOLOGIC VARIATIONS OF *B. megatherium* AT DIFFERENT STAGES OF GROWTH.

2 hours, so that the drawing of the two-hour sample serves to indicate the character of the cells used for seeding. These were taken from a rather old culture on dextrose agar. It will be observed that three kinds of cells were present in the seeding, namely, spores, sporangia, and cells of irregular form containing numerous granules. As growth proceeded these all rapidly became transformed into long cylindrical cells, practically free of granules. The spores germinated by simply gradually swelling and assuming elongated form. The same thing happened to the irregular cells that were present in the seeding, so that by six hours there were to be found only these long, sausage-shaped, relatively hyaline cells. As growth proceeded these long, clear cells became smaller and more and more filled with granules. The number of spores present in the various samples was somewhat irregular, probably due to variations in the distribution of spores throughout the agar slant, as all of these preparations were taken from the same agar slant. The large number of free spores present, for instance in the twelve hour sample, were probably ungerminated spores that had been present in the original seeding, and there is no evidence of active spore formation again until after thirty-six hours. By this time it will be noticed that all of the cells are relatively small; the difference between the thirty-six and the six-hour sample is, for instance, quite striking. With increasing age, more and more spores develop within the cells, and the cells become more and more irregular in form and filled with granules, until when the culture is a week old we have nothing left but free spores and bizarre, irregular, granular involution forms. Thus it will be seen that at all stages there is a continuous and regular metamorphosis taking place.

For the present we will concern ourselves with the changes which take place during the early stages of growth, with the development of the large, clear cells characteristic of the active growth phase. Variations in the size of the cell have been studied in micro-colonies by continuous observation, and in a series of cultures on agar slants and in broth, from samples removed at regular intervals of time during growth.

The micro-colonies were prepared by inoculating a loopful of culture into a tube of molten agar. A loopful of this agar sus-

pension was then placed on a cover glass, which was inverted over a hanging drop slide and sealed with vaseline to prevent evaporation. These were incubated in an electric warm stage at 30°C. With the low-power lens a cell or small group of cells was found. This was then focused upon with the oil immersion lens, and watched continuously for some hours, the progress of events being recorded by camera lucida drawings. Such micro-colony observations are not easily made, for if the preparation is heavily seeded, so that the cells are easy to find, then the growth rate is low and the morphologic variations are slight. If, however, the preparation is lightly seeded, so that there are only a few cells in the whole preparation, then these cells are difficult to find, and in such preparations isolated cells frequently failed to grow for some hours. Many such preparations were made and observed at intervals all day long, without any evidence of growth being seen, and then on the following morning it was found that a good sized colony had developed. Most successful results were obtained when small groups of cells, generally in chains, were found well isolated from any others.

The results of such continuous observations of growing micro-colonies are presented in Figures 10, 11, and 12. The first developed from two cells, both of which commenced to grow shortly after the observation was begun and grew at approximately the same rate throughout the period of observation. Over three hours was required for the first cell division to take place. During this period, however, the cells had greatly elongated, to considerably more than twice their original length, and the contained granules had gathered at the poles of the cell, and had perhaps also decreased somewhat in number. After the first cell division, which occurred approximately simultaneously in the two cells, the four resulting cells again increased markedly before they divided, increasing once more to more than twice the size at the preceding cell division. The second micro-colony developed from three cells, of which only one grew, the other two showing no changes in morphology during the period of observation. The cell which grew showed its first evidence of growth in an increase in length, with, at the same time, a decrease in the number of granules and an accumalation of these granules at that end of the cell which was not in contact with its sister cell. Cell division oc-

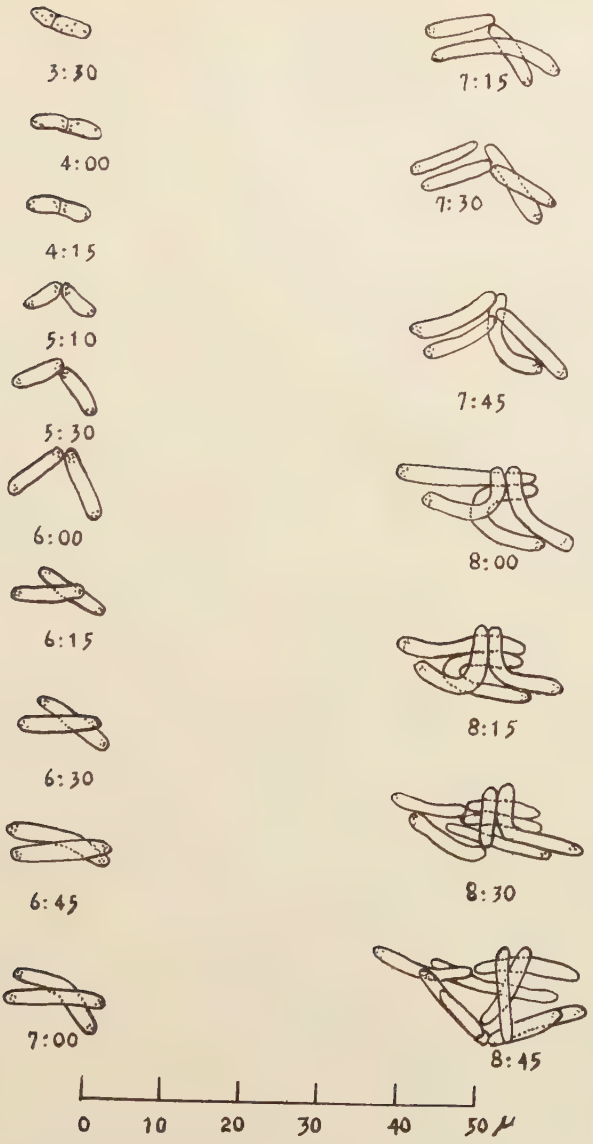

FIG. 10. MORPHOLOGIC VARIATION IN A GROWING MICROCOLONY OF *B. megatherium:* MICROCOLONY I.

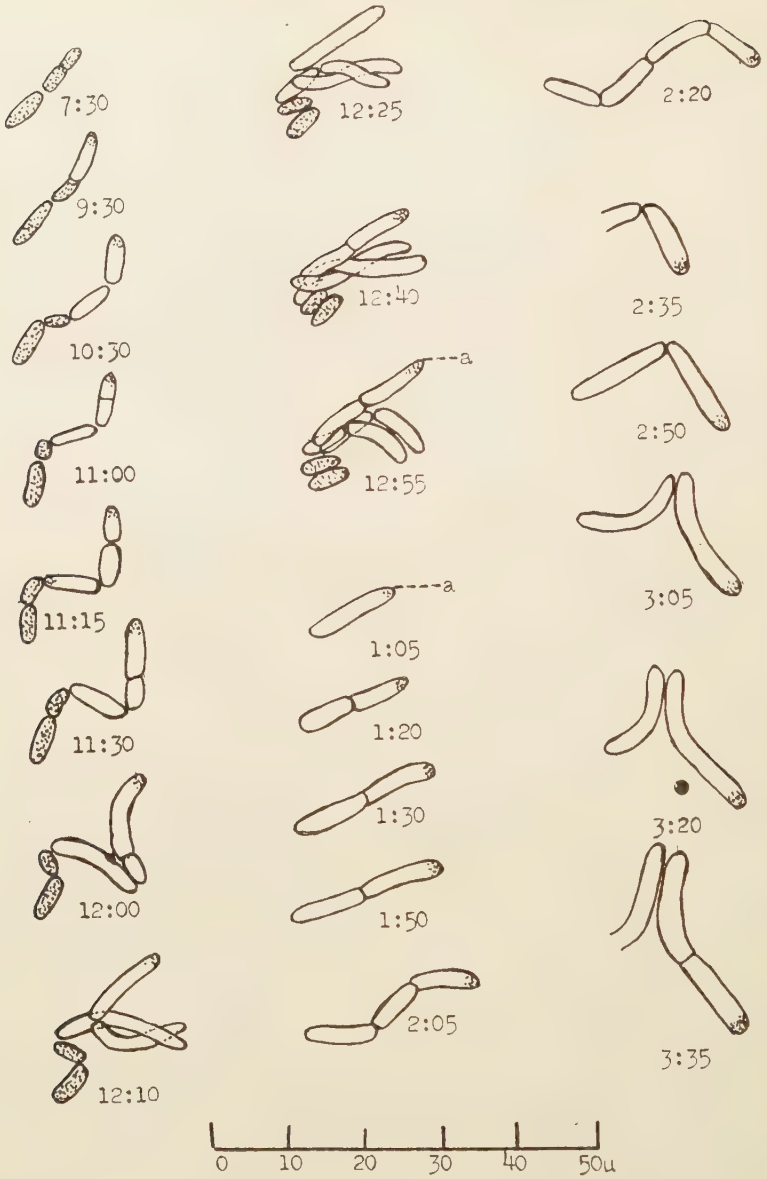

FIG. 11. MORPHOLOGIC VARIATIONS IN A GROWING MICROCOLONY OF *B. megatherium:* MICROCOLONY II.

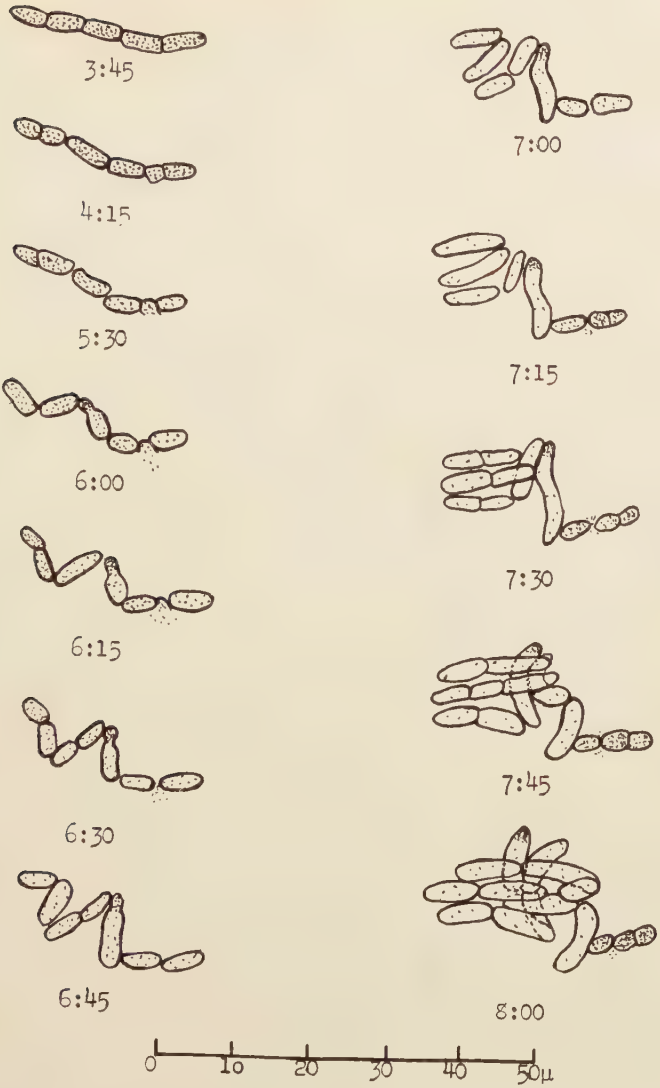

FIG. 12. MORPHOLOGIC VARIATIONS IN A GROWING MICROCOLONY OF *B. megatherium:* MICROCOLONY III.

From "Morphologic Variations of Bacteria in the Lag Phase." Reproduced through the courtesy of the Journal of Infectious Diseases (1926, **38**, 54).

curred shortly after this increase in length, and in repeated cell divisions it is observed that the cells become progressively longer before each division. The new cells formed are free of granules, but the granules present in the original tip cell remain, and can be seen throughout the subsequent periods of observation. After some five hours growth, the number of cells began to increase greatly so that it was difficult to follow individual cells, but the original tip cell was still easily distinguished, and repeated divisions of this portion of the colony were followed for several hours longer.

In studying these developing micro-colonies, two rather interesting features are observed, other than the changes in size and form of the cells. One particularly evident in the second micro-colony, is the apparent tendency for growth to be restricted to the end of a chain of cells as long as the cells remain in a chain. This is reminiscent of the terminal growth of the filaments of mycelium in molds. It is not clearly distinguished in micro-colonies or in cultures from solid media, where the chains, during the period of active growth at least, quickly break up; but in preparations from broth cultures this is very frequently clearly evident; long chains of cells are formed in which the two terminal cells are found to be much longer than other cells in the chain. The second feature is the mechanism by which the chain is broken and the cells are separated. This is particularly evident in the first two micro-colonies. It is seen that after a cross wall has formed, the two cells bend at right angles, so that the cells are finally torn apart by the leverage of their pressure against each other. This mechanism has been previously described in spore forming bacteria. It is also noteworthy that the actively growing cells are free of granules, though those granules which were present in the original cell at the beginning of growth may be retained at the tip, which serves to identify it through the subsequent periods of observation.

The third micro-colony developed from five cells. It will be observed that for a period of three hours there was practically no evidence of growth. One of the cells developed a cross wall, and one of the daughter cells so formed proceeded to disintegrate. Another became deformed, developing a constriction at one end, with an accumulation of granular material in the constricted portion. At the

end of three hours, however, some of the cells showed evidences of growth by an increase in size and a decrease in the number of granules contained in the cells, and at an increasingly more rapid rate this process continued, the cells becoming longer and freer of granules; and after they did so they also divided. Three of the cells remained throughout the period of observation without any signs of growth, actually becoming smaller and more densely packed with granules.

It is evident from these micro-colonies that two kinds of growth are occurring—an increase in the size of the cells, and an increase in the number. A true measure of the rate of growth, then, is obtained only by considering both, i.e., by obtaining the product of the number and size of the cells. The relationship of these conditions to each other is shown graphically in Figure 13, and the values from which these graphs have been plotted are presented in Tables III, IV, and V. In order to plot the three quantities, namely the number of cells, the mean length of the cells, and the total cell length, on the same scale, they have been converted to index numbers, the index in each case being the mean value for the entire period of observation; that is, the absolute number of cells has been converted into a percentage of the average number of cells, and the same process has been carried through with the other two values. In the first colony, where the two cells both grew simultaneously, the curve for the average length of the cells is interesting. It will be noted first of all that there was a definite lag in this curve, active increase not being apparent until after 180 minutes; that the cells showed no change in size for a short period preceding each cell division; that the size of the cells after cell division was greater than the size at the beginning of observation, indicating that the increase in size is definitely more than twice; and that the size attained after the first cell division was greater than the size just previous to the first cell division. There is, then, a sort of rhythmical character to the curve for cell size. The cells increase to a certain length, divide, then grow again to reach a still higher length, divide, and so on. The increase in size of course precedes the first cell division, so that if we plot the absolute rate of growth in terms of total cell length, the lag is very slightly shorter than the lag in the curve for numbers of cells, but

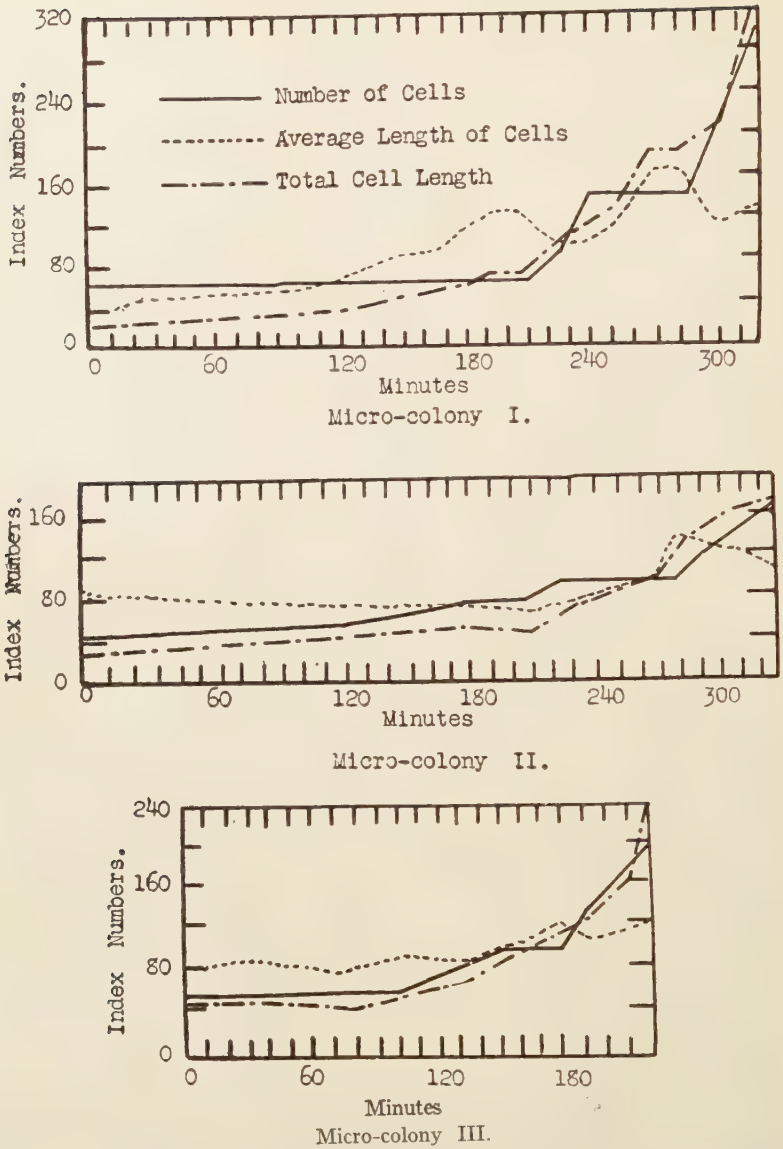

FIG. 13. GROWTH CURVES AND VARIATIONS IN CELL SIZE IN GROWING MICRO-COLONIES OF *B. megatherium*.

From "Morphologic Variations of Bacteria in the Lag Phase." Reproduced through the courtesy of the Journal of Infectious Diseases (1926, **38**, 54).

not enough so to be of great significance. In the second colony only one of the inoculated cells grew; the others actually decreased somewhat in size. We have again a long lag period in the curve for cell size, during which they actually decreased somewhat. In the third micro-colony the changes in cell size are not nearly so pronounced. It will be remembered that this colony started from five cells, of which three grew, and as will be seen later, the tendency is for the change in cell size to be less with increased seedings.

From these micro-colonies we may draw the following tentative conclusions. The increase in number of cells is accompanied by an increase in size. This is apparently more marked with the small seeding (first colony, starting from two cells) than with the larger seeding (third colony, developing from five cells). The lag period before the beginning of cell multiplication is not entirely occupied by a transformation of the mature cells to large growing cells. There is also a lag phase in the curves for cell size. Not all of the cells respond equally to the new medium, some remaining dormant for considerable periods of time. There does, therefore, occur a selection of a rapidly growing strain in some cases. The cells which remain dormant may continue to change in morphologic characters as they would have if they had remained in the old medium, becoming smaller in size, distorted in form, or dying and disintegrating.

Further studies have been made in a culture grown on agar slants (designated Culture I in the tables), following the technique described in Chapter III. The agar slant tubes were inoculated from an agar culture which had been transferred every twelve hours for several days to get rid of spores, since it was desirable to study the morphologic variations during the early stages of growth uncomplicated by spore germination. In all of these studies of *B. megatherium* the cultures used for seeding were free from spores as far as could be determined by microscopic examination. Samples were removed every half hour for the first twelve hours of growth, every hour for the next twelve hours, every four hours for the second twenty-four-hour period, and again at sixty and seventy-two hours. Cell counts were made beginning with the first half-hour period, and are presented in Table VI. The number of free spores was observed after spore formation commenced, and is recorded in the same

table. Not a sufficient number of cells was obtained before the first two and one-half hours to make quantitative studies of the size of the cells, but from then on such observations of morphologic

Fig. 14. Growth Curve and Variations in Size of Cells of *B. megatherium*: Culture I.

characters were made. The results were subjected to statistical analysis, and are presented in Table VII. In Figure 14 the rate of growth, as indicated by the logarithms of the number of cells, is plotted

for the first sixteen hours, at which time growth had practically ceased. It will be noticed that there was a lag period of about four hours, after which time a maximum growth rate was quickly attained, which continued for another hour, and then the growth rate began to slow up, and there was a gradual decrease until the end of the growth period.

In this same graph the mean length of the cells is plotted. It will be seen that this curve also presents a latent period, which is, however, shorter, ending at two and one-half hours. Then the cells rapidly increase in length, and the increase is surprisingly great, the maximum size reached at five and one-half hours being approximately six times that of the original cells introduced into the medium. Following this increase the cells as rapidly decrease again, and at the end of nine hours they have practically reached the original size. This period corresponds fairly closely with the point of inflection in the growth curve between the period of active growth and the resting period. From then on the size of the cells shows slight fluctuations, but is relatively constant throughout the remaining period of observation. Although both curves show little fluctuations due to experimental error, so that it is impossible to use the data without smoothing for purposes of studying correlation, it is quite evident from the curves that there is a definite correlation between the size of the cells and the rate of growth. The size of the cells increases as long as the growth rate is increasing. It reaches a maximum at a point corresponding to the point where the rate of growth changes from positive to negative acceleration, and returns to a minimum at a point corresponding with the point of inflection between the phase of growth and the resting phase.

It will be noted that while the curve for cell size shows a lag, this lag period is shorter than the lag period in the curve for cell numbers, and that, therefore, a change in cell size precedes actual cell division, but that the lag period is not entirely occupied by this change in cell size. Since the cells are increasing both in size and in number, a true measure of growth is obtained only by plotting the total cell length (assuming, as will be shown later is nearly the case, that the cells do not change much in diameter, and that the length of the cells is therefore a true measure of their size). Since in the

later phases of growth, the increase in number of cells is much greater than the increase in size of cells, such a curve of total cell length does not differ much from the curve of the number of cells, except during the early hours of growth. In Figure 15 the number

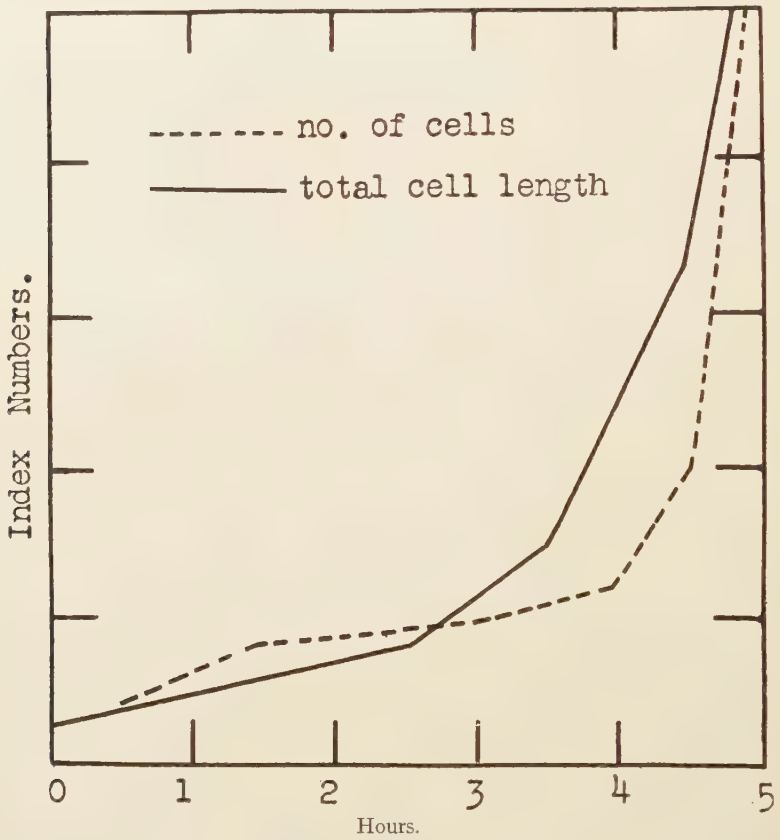

FIG. 15. COMPARISON OF GROWTH CURVES BY CELL COUNTS AND BY TOTAL CELL LENGTH IN THE LAG PHASE. *B. megatherium*, CULTURE I.

of cells and the total cell length are plotted side by side. It will be seen that the latter curve presents a somewhat shorter latent period and a more gradual acceleration than the former.

A number of interesting facts are brought out by studying the frequency distribution curves of the cells with regard to their size

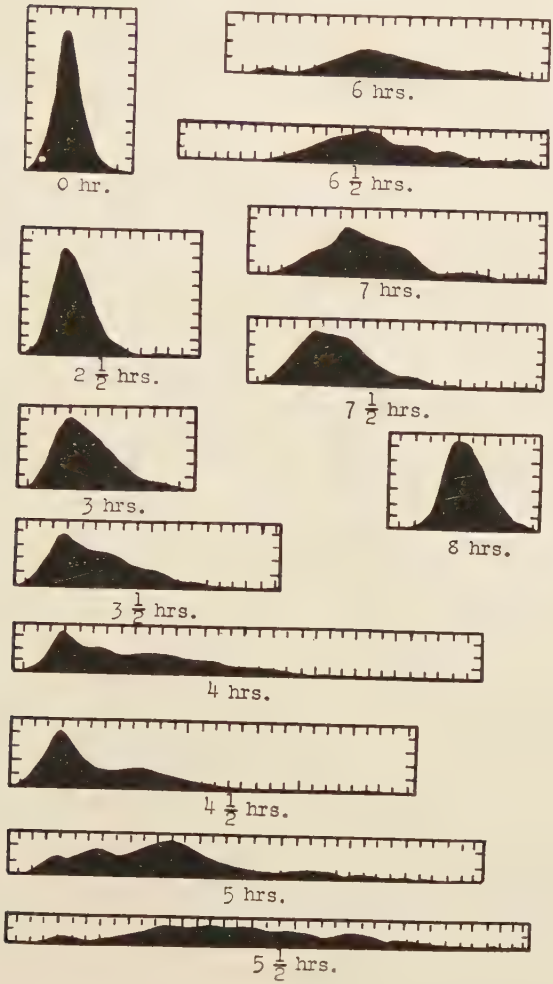

FIG. 16. FREQUENCY DISTRIBUTIONS OF CELLS ACCORDING TO LENGTH AT DIFFERENT STAGES OF GROWTH. *B. megatherium*, CULTURE I.

From "Morphologic Variations of Bacteria in the Lag Phase." Reproduced through the courtesy of the Journal of Infectious Diseases (1926, **38**, 54).

at various periods of growth. These frequency distributions for the first twelve hours are presented in Figure 16. The material used for seeding (zero hour) presented a practically symmetrical distribution, with a relatively narrow range and a high mode. As growth progressed, the form of this distribution curve shows progressive changes. At the end of two and one-half hours, the range is distinctly increased, the mode is lower, and the curve has become somewhat skewed. This increase in range, decrease in height of the mode, and increase in skewness becomes continually greater in the succeeding curves. At the end of four hours there is an apparent division, appearing as though a new mode were being established. This is more evident in the four and one-half hour curve, and in the following hour, when the change in size was greatest of the entire period of observation, this new mode becomes higher; the original mode practically disappears; and we now have established a somewhat irregular curve with a long base line and a low mode. In the sixth hour curve the last remnants of the original mode are seen for the last time. From then on the curve, though irregular, increases in its base line, increases its height, and at the end of eight hours is again approaching the form of the original curve. This was finally reached at twelve hours, and from then until the end of the period of observation the frequency distribution curves show no significant variations.

It is clear, then, that the increase in size of cells is also accompanied by an increased variation in size, which is quite evident by observing the standard deviations presented in Table VII. This seems to be a general biological phenomenon which has been observed, for instance, from the study of the growth of humans; the coefficients of variation are higher in growing children than in adults. The skewness and the apparent bimodality of the curves during the period of increasing size, that is during the period of accelerating growth, are of particular significance. They indicate that not all of the inoculated cells commence to increase in size at once, for if they did so, and all increased proportionately, the curve would simply move in the direction of greater size without changing in symmetry, but if at first a few, and then increasingly greater numbers of cells commenced to grow and elongate, there would be produced just such a series of changes in distribution as has been described,

namely increased range and dispersion and skewness. But as long as this process is continuous, while there would be produced a gradual upset of the whole curve with the final development of a new

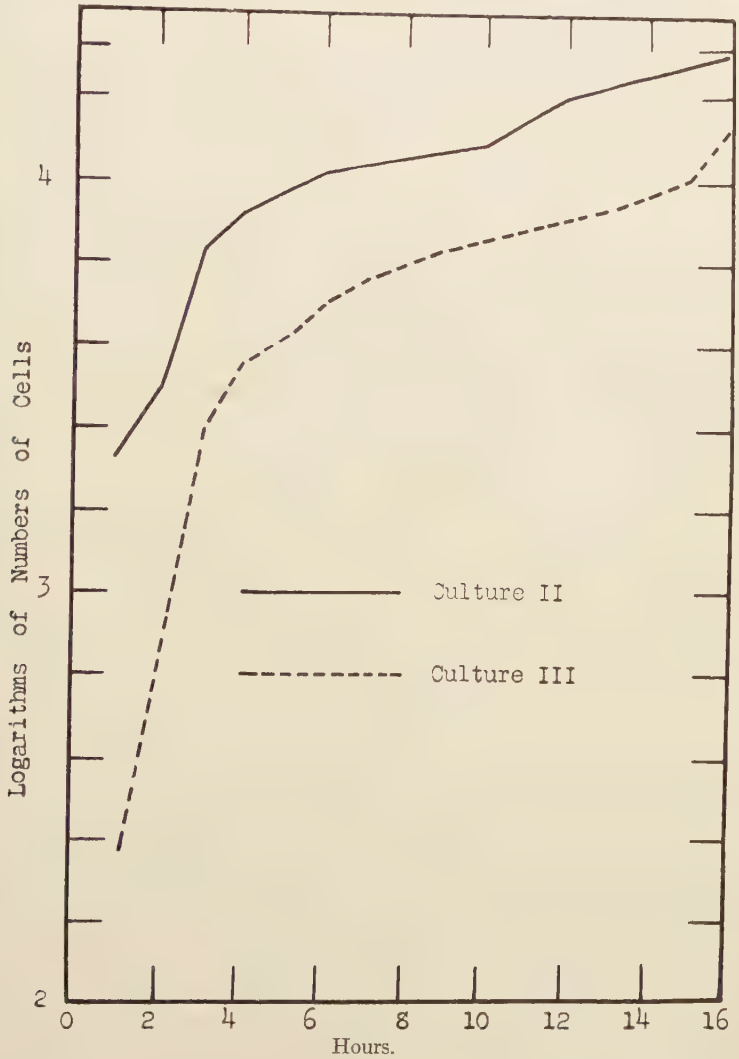

FIG. 17. INFLUENCE OF SIZE OF SEEDING ON GROWTH CURVES OF *B. megatherium*: CULTURES II AND III.

mode, there would not occur distinct bimodality such as is apparent here. If, however, out of the inoculated cells, one group started to grow, while the others remained dormant, bimodality, with a new mode growing at the expense of the old one, would result.

Further studies were made in broth cultures, and these were run in parallel series with varying sizes of seeding, in order to deter-

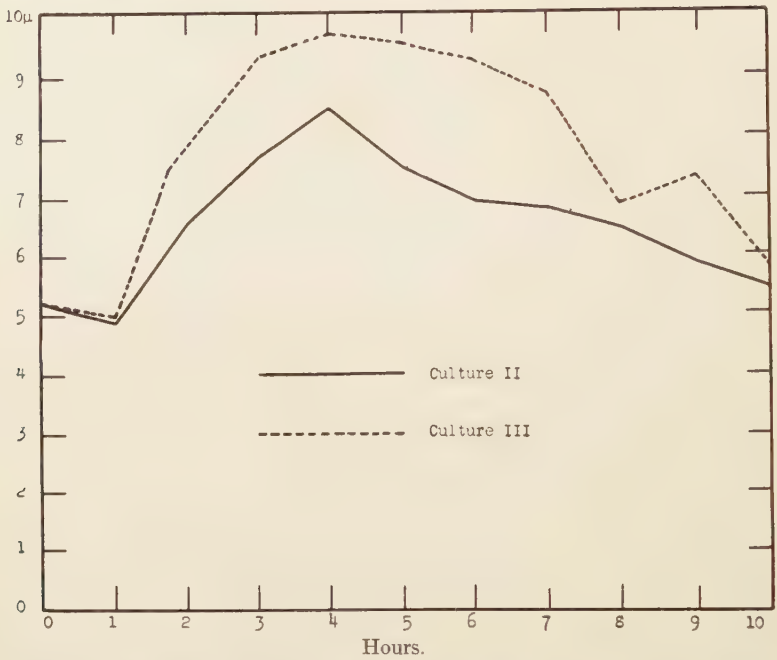

FIG. 18. INFLUENCE OF SIZE OF SEEDING ON LENGTH OF CELLS OF *B. megatherium:* CULTURES II AND III.

mine more clearly the influence of the rate of growth upon the size of the cells. The first series, marked Cultures II and III, were inoculated simultaneously from a seven-hour agar slant culture, again after transplanting every twelve hours for several days to get rid of spores, Culture III being seeded with one-tenth as many cells as Culture II. Since this culture started from a very young one, no pronounced lag phase was noted. The experiment was therefore repeated in Cultures IV, V, and VI, using a twenty-hour broth

culture for inoculation, hoping thus to get a lag period uncomplicated by spore germination. This hope, however, was not fulfilled, lag being absent. Culture V received one-tenth, and Culture VI one-hundredth as many cells as Culture IV.

From these broth cultures it is quite apparent that the changes in cell size are not nearly so extensive as those observed in the preceding agar culture; but also the rate of growth is much lower. In the agar culture (Culture I) the mean generation time for the first twelve hours was sixty-eight minutes; in Culture II, it was 317 minutes; in Culture III, 153 minutes; Culture IV, 223 minutes; Culture V, 111 minutes; Culture VI, 78 minutes. The maximum size attained in these cultures was as follows: Culture I, 19.8μ; Culture II, 8.5; Culture III, 9.7; Culture IV, 7.5; Culture V, 8.0; Culture VI, 8.6. It is apparent, then, that the size attained depends upon the actual rate of growth in the culture, the shorter the generation time the greater being the length of the cells; but since these experiments were not all run under exactly the same conditions, this correlation is of course not a perfect one. The relationship of the size of the cells to the rate of growth is more clearly apparent by consulting the graphs in Figures 17 and 18 (the data for which may be found in Tables VIII, IX and X), which show the growth curves and the curves for the size of cells in Cultures II and III. The more heavily seeded culture (II) shows a lower growth rate, as indicated by the slope of the curve during the logarithmic growth phase, than Culture III, and correspondingly the curve for cell size reaches a lower maximum and declines more rapidly than in the culture (III), which was seeded with one-tenth as many cells. The same thing was found to be true in Cultures IV, V and VI (data for which will be found in Tables XI, XII, XIII, XIV), the curves for cell size being somewhat irregular, but again the maximum was higher in Culture VI, which was most lightly seeded, and this size was maintained for a longer period of time.

Corresponding with the difference in actual size of the cells in these broth cultures, there was found a distinct difference in the degree of variation in size. For Cultures II and III the frequency curves are presented in Figures 19 and 20. It is apparent at once that in neither culture were the changes in the form of the distribu-

tion curve as pronounced as was found to be the case in the agar culture, which had a much higher growth rate. In Culture II there

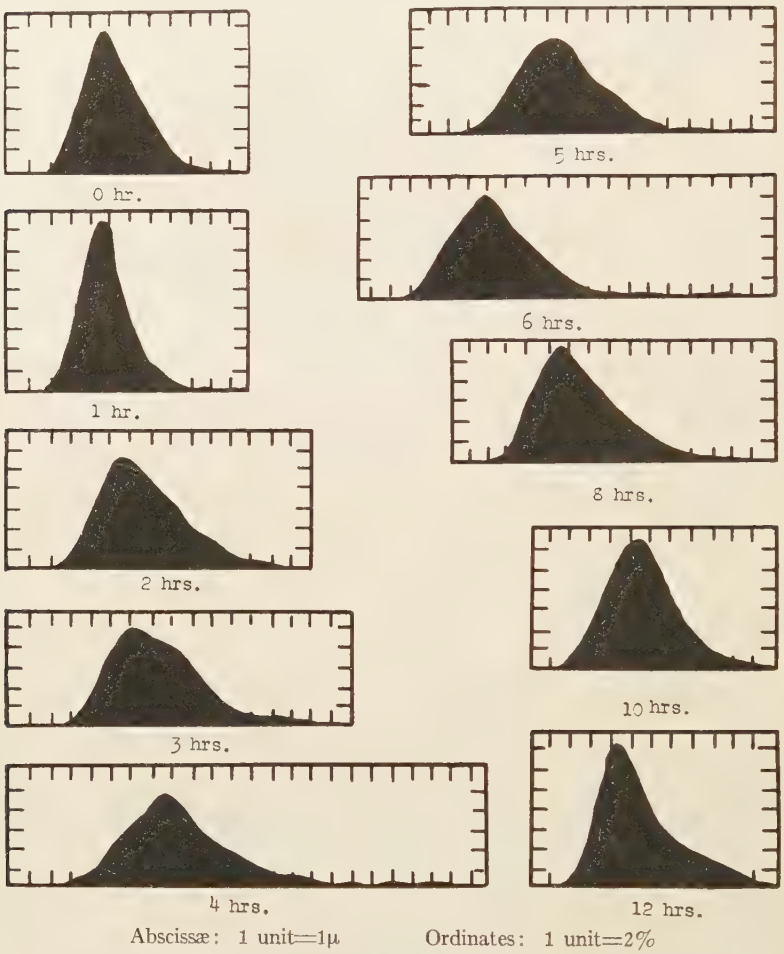

FIG. 19. FREQUENCY DISTRIBUTIONS OF CELLS ACCORDING TO LENGTH AT DIFFERENT STAGES OF GROWTH, *B. megatherium*, CULTURE II.

is very little change other than skewness and a shifting of the mode. In Culture III the skewness, shifting of the mode, and extension of the base are still more apparent, and in the three hour sample,

approximately the period of maximum growth rate, there is some tendency to bimodality. The same things were observed in Cultures IV, V, and VI; with the decreasing sizes of seeding, that is with

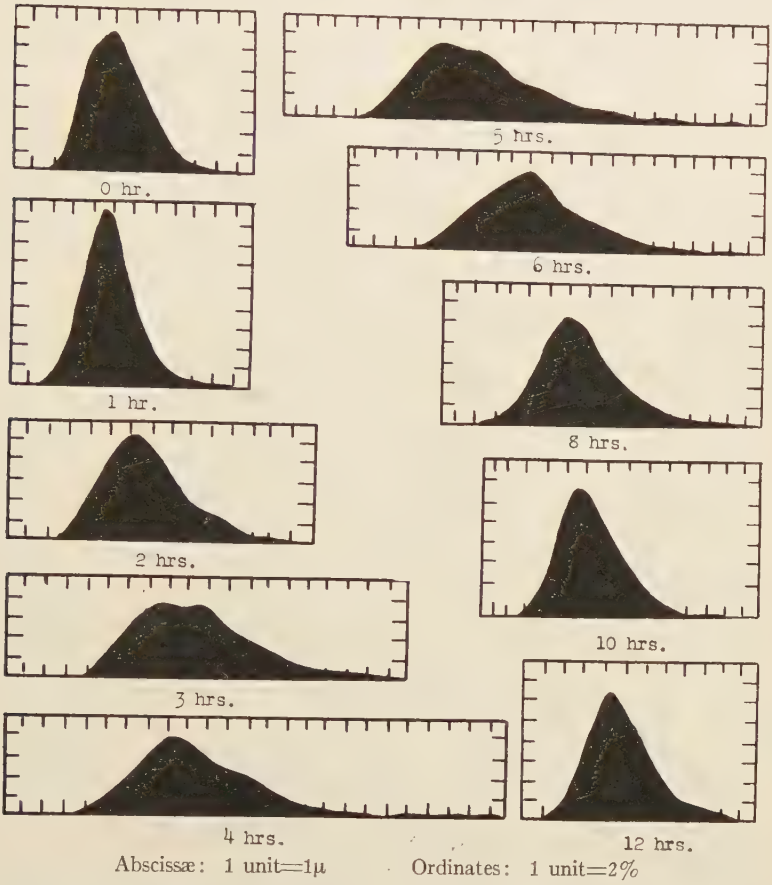

FIG. 20. FREQUENCY DISTRIBUTIONS OF CELLS ACCORDING TO LENGTH AT DIFFERENT STAGES OF GROWTH, *B. megatherium:* CULTURE III.

increasing growth rates, there was greater change in the form of the distribution curves, and only in the last of these three was there any evidence of bimodality in the curves.

From these quantitative studies in mass cultures, the same con-

clusions can be drawn as from the micro-colonies, though perhaps the evidence is more clear, namely that the period of maximum growth is accompanied by an increase in size of the cells, and that this is correlated with the rate of growth, being greater with lightly seeded cultures than with more heavily seeded ones. The maximum size corresponds with the moment of maximum growth rate. There is a lag period in the curve for cell size, and the lag period in the curve for cell numbers is therefore not entirely occupied by the transformation of the small cells to large, growing ones. But the cells do increase in size before they begin to divide, and if we consider growth both from the standpoint of cell size and cell numbers, that is the total growth in the mass of proptoplasm, then the lag phase is correspondingly shortened.

Not all of the inoculated cells grow at the same rate; first a few and then more and more cells commence to grow, manifesting their growth by increase in size; and by studying the frequency distribution of the cells according to their size, we can demonstrate this variation in growth potentiality of the cells inoculated into the medium. This degree of variation is greater with smaller seedings, with very small seedings there being a pronounced tendency for bimodality to develop in the distribution curves, indicating that under such circumstances there does occur, as was postulated by Penfold, a selection of a rapidly growing strain from the inoculum. That this depends upon the size of the seeding will be apparent after a moment's thought. If we inoculate a culture with two cells, there are two possibilities: First, that both of these will grow at the same rate; second, that they will grow at different rates. Because of the tendency to variation inherent in living organisms, the chances are in favor of the second possibility. If we have three cells, then the chances are that there will be three different growth rates, and by steadily increasing the number of cells we increase the possibility of having a perfectly graded series of cells with regard to their growth rate when transferred to a new medium. In a culture thus inoculated with a large number of cells those cells which have the maximum growth potentiality will of course start to grow soonest, but they will be followed quickly by the cells having the next highest growth rate, and so on; and we will thus have a perfectly graded series

of cells starting to grow one after the other, and so producing a skewed but continuous frequency distribution curve. If, however, our seeding is small, the chances will be greater for there occurring gaps between the cells having the maximum growth rate and those

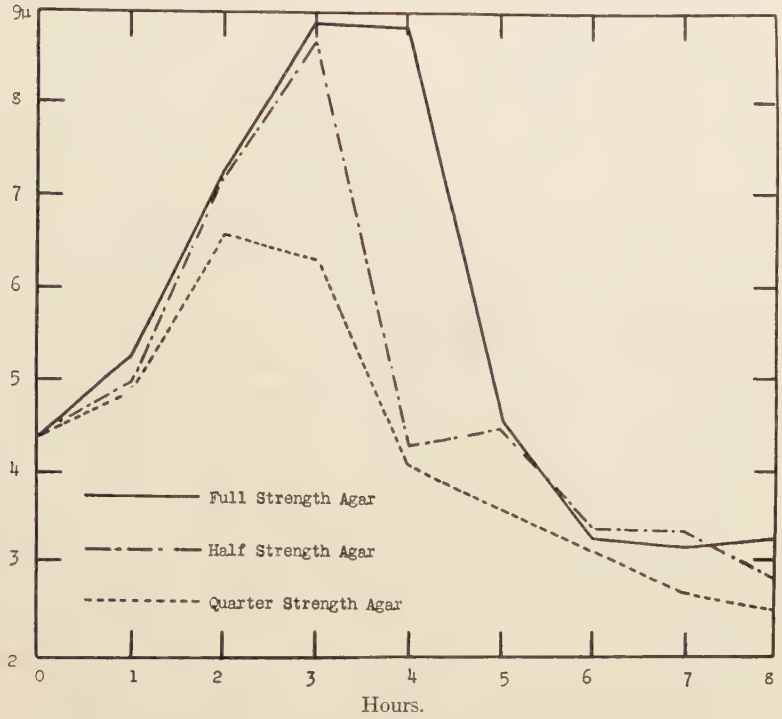

FIG. 21. INFLUENCE OF CONCENTRATION OF NUTRIENTS ON LENGTH OF CELLS OF *B. megatherium*.

From "The Influence of the Concentration of Nutrients on the Size of the Cells of B. megatherium." Through the courtesy of the Society for Experimental Biology and Medicine (Proceedings, 1921, **19**, 132).

cells which do not grow at all, and the smaller the size of the seeding, therefore, the greater will be the tendency for the frequency distribution curves to become broken into two or more modes.

The relationship of the size of the cells to the rate of growth has been further studied in cultures inoculated with the same number

of cells but into media varying in the concentration of foodstuff present. Ordinary nutrient broth was diluted to one-half, to one-fourth, to one-eighth, and to one-sixteenth of its original concentration, and these batches of broth were made up into agar slants as described in Chapter III. All of the cultures were then simultaneously inoculated from the same suspension of cells of *Bacillus megatherium*. Cell counts were not made from these cultures, so that it is not possible to determine the actual rates of growth, but from the data presented in Chapter II we know that the rate of growth must have been proportionately higher with increasing concentrations of nutrient substances in the medium. The results of the measurements of cell length are presented in Table XV, and curves for the first three dilutions, full-strength, half-strength, and quarter-strength medium, are presented in Figure 21. It will be noted from Table XV that the results were not perfectly consistent, the eighth-strength medium reaching a slightly higher maximum than the quarter-strength; but that in general the maximum size attained was directly proportional to the concentration of foodstuff in the medium. Since observations were made only hourly, it is quite evident that the peaks of the curve for full-strength and quarter-strength agar would probably have been higher if observations had been made more frequently, and this fact probably explains the apparent inconsistency in the maximums which can be observed in Table XV.

Frequency distribution curves for three of these cultures are shown in Figure 22. They show in general the same characters as have been previously described; with increase in the rate of growth dependent upon the concentration of nutrient in the medium there is a greater degree of variation in the size of the cells, greater extension of the base line, and flattening of the curve. Only in the full-strength medium is there a tendency to split into two groups distributed about separate modes. This is somewhat difficult to explain if the reasons given above for bimodality are accepted, but it might be suggested that an increase in strength of the selecting agent (nutrients) might as well increase the tendency to bimodality as a decrease in the number of inoculated cells. At any rate it is clear that increasing growth rate from whatever cause is accompanied

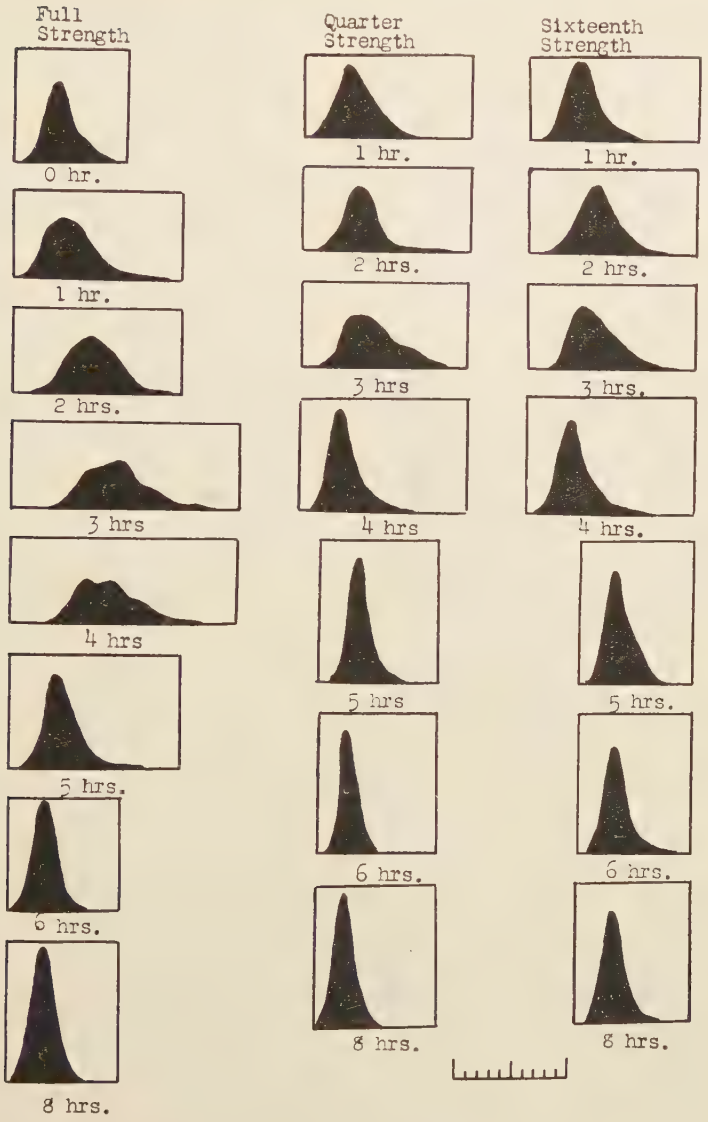

FIG. 22. INFLUENCE OF CONCENTRATION OF NUTRIENTS ON FREQUENCY DISTRIBUTION OF CELLS ACCORDING TO LENGTH, *B. megatherium*.

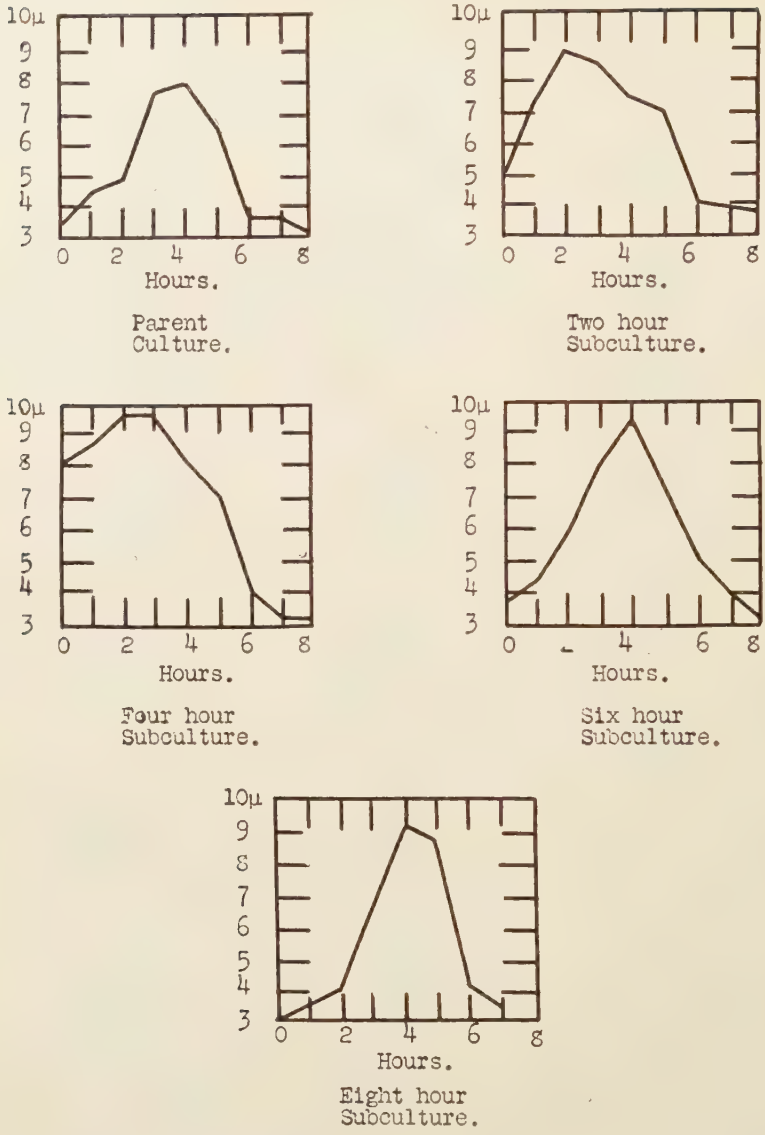

FIG. 23. INFLUENCE OF AGE OF PARENT CULTURE ON SIZE OF CELLS, *B. megatherium*.

by increased variation in size as well as by increased size of the cells.

It will be remembered that Chesney demonstrated that the rate of growth is also dependent upon the age of the cultures used for inoculation, and this factor has also been studied with regard to the size of the cells. A series of agar slant tubes prepared as described in Chapter III were inoculated with a culture of *Bacillus megatherium;* samples were removed every hour and measures of cell length made. After two, four, six, and eight hours, subcultures were made onto other agar slant tubes prepared in the same way. An attempt was made to keep the size of the seeding constant by preparing suspensions of as nearly as possible the same degree of turbidity, as determined by inspection. The results are presented in Figure 23, which shows the mean length of the cells plotted against the age of the culture. It will be seen that the parent culture showed in general the same type of curve as has previously been described. There was a slight latent period, after which the cells increased in size, reached a maximum, decreased rapidly and then more slowly until they reached the original size. In the culture transplanted from the parent culture when it was two hours old, the cells continued to increase in size at the same rate as in the parent culture, but reached a somewhat higher maximum and maintained their large size for a somewhat longer period of time. In the culture transplanted from the parent culture when it was four hours old, the cells showed a somewhat further increase in size, but at a somewhat lower rate, and the increase in size of the four-hour subculture over the two-hour subculture was less than the increase of the two-hour subculture over the parent culture. By continually subculturing during the maximum growth rate, then, we can continue to increase the size of the cells, but this increase is not proportional, the amount of increase being smaller with each succeeding subculture, so that there is a definite limit to the size attainable. In the culture transplanted from the parent culture at the end of six hours, the cells immediately started to increase again, and reached a somewhat higher maximum than in the parent culture; but when transplanted at the end of eight hours, that is at a time when the cells in the parent culture had returned to their minimum

once more, the cells in the new culture again showed a lag before they commenced to increase, and reached practically the same maximum as in the parent culture, the curve having practically the same form as that of the culture from which the seeding was taken.

Here, then, is further evidence that the size of the cells is correlated with the rate of growth. When a culture is transplanted at a period during which the cells are increasing in size, they continue to increase in size in the new medium; just as Chesney found when a culture is transplanted during a period of maximum growth rate, it continues to grow at a maximum growth rate in the new medium. When the cells are transplanted during the period of decreasing size, they immediately recover and increase again in the new medium, but if transplanted at the time when they had reached their minimum size, corresponding with the resting period or period of no growth, they show a lag in the new medium, just as Chesney found there was a lag in the curve for rate of growth.

Frequency distribution curves of these cultures also support the previous observation that the degree of variation in cell size is correlated with the actual size attained, that is with the actual rate of growth in the culture. The parent culture showed the extension of the base line, and flattening of the peak during the period of maximum cell size, as was observed in the previous cultures. In the two-and four-hour subcultures, this extension and flattening was increasingly greater, but in the eight-hour subculture the frequency curves were practically identical with those in the parent culture.

CHAPTER V

THE SIZE AND FORM OF THE CELLS OF THE COLON BACILLUS

In the studies of *Bacillus megatherium* immediately preceding, the only measure of size considered was the length of the cells. There were several reasons for this, the most important being that the diameter of the cells is so small that by the technique used it was impossible to make measurements of the thickness at all comparable in accuracy with the measurements of length. It was quite apparent, however, that while there was a degree of correlation between the length and diameter of the cells, the longer cells being somewhat thicker, nevertheless the increase in thickness with increasing length was by no means proportional; that is, the longer cells were relatively much more slender, and it was assumed, therefore, that measurements of length alone would be sufficient to indicate the character of the variations in size which were taking place. It was found desirable, however, to make some observations on this change in the relative proportions between length and thickness of the cells, that is to obtain some sort of a quantitative expression of the variations in the form of the cells which were taking place.

This was done with a culture of *Bacillus coli,* using photomicrographs projected so as to give a final magnification of 30,000×. From the tracings of the projected cells the area was measured, as well as the length along the major axis of the cell and from these two measurements the quantitative expression of the form of the cell was obtained by dividing the area of the projected image by the length squared. The quantity so obtained, which I have designated the area-length index, is an abstract measure of form, regardless of the size of the cell. It will have its highest value if the cell from which the tracing is made is a sphere, that is the image being circular. As the length increases in proportion to the width, this value becomes progressively smaller.

Such a measure suffices to express accurately the form of the

cell as long as this is symmetrical about both axes. It will be at once apparent, however, that this does not serve if the cell is at all asymmetrical. If, for instance, we have bulgings or constrictions or lateral projections, such unusual cell forms might have the same area-length index as a cell which was quite uniform throughout. With the early stages of growth, however, such asymmetrical cells are exceedingly rare, and therefore for this stage the area-length index serves very well to indicate the variations in form of the cells. By the use of this measure, together with the length of the

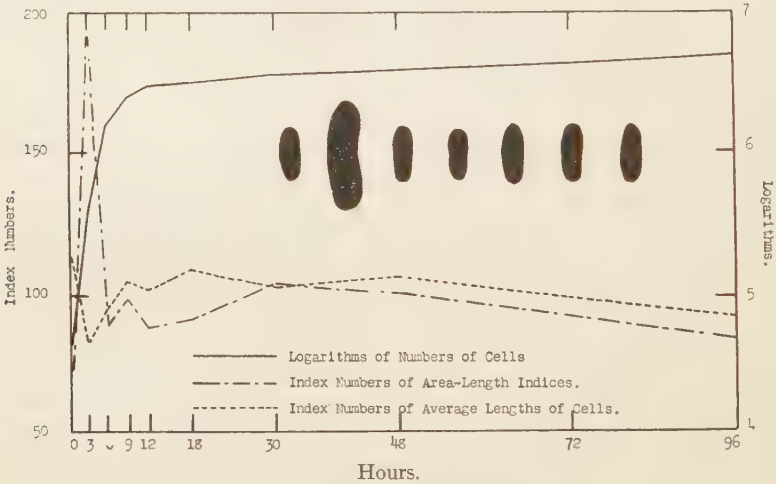

FIG. 24. GROWTH CURVE AND MORPHOLOGIC VARIATIONS OF A CULTURE OF *B. coli.*

From "A Statistical Study of the Form and Growth of Bacterium coli." Reproduced through the courtesy of the Society for Experimental Biology and Medicine (Proceedings, 1923, **21**, 215).

cells, we can therefore follow consecutive changes both in the size and the form of the cells, and correlate these with each other, as well as with the rate of growth.

The organism used was an old laboratory strain, a typical colon bacillus. The culture was inoculated onto agar slants, following the technique described in Chapter III. Samples were removed for counting and measurement every three hours for the first twelve hours, and again at eighteen, thirty, forty-eight, seventy-two, and ninety-six hours.

Figure 24 shows in a general way the results of these observations. In this graph the logarithms of the number of cells indicate the rate of growth; the average length of the cells and the average area-length index having been converted to index numbers, using as the index the mean value of each for the entire period of observation. The data are given in Tables XVII and XVIII. As with *Bacillus megatherium,* there was noted a very marked increase in the length of the cells during the period of active growth, reaching its maximum

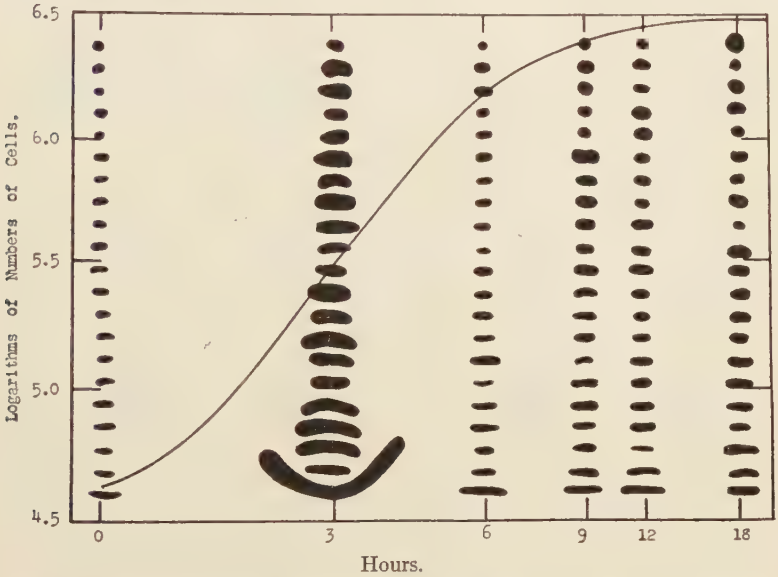

FIG. 25. REPRESENTATIVE CELLS FROM A CULTURE OF *B. coli* AT DIFFERENT STAGES OF GROWTH.

probably again at the point where the growth curve changes from positive to negative acceleration. It will be noted, however, that the curve for area-length index during the early hours of growth is practically the reverse of the curve for length of cells; that is, as the cells increase in length they decrease in area-length index, the longer cells being relatively more slender, even though they were absolutely somewhat thicker. In this graph the average size and form of the cells is indicated by tracings through the major image of composite photographs of the cells prepared as described in

Chapter III. They illustrate well the increase in size during the period of active growth. After growth has come to a standstill and the cells have returned to their original size, subsequent variations are slight and probably of no significance.

This negative correlation between the length and area-length index of the cells will be perhaps more apparent by an inspection of Figure 25, in which every tenth cell in the array of 200 from each sample has been superimposed in a column upon the growth

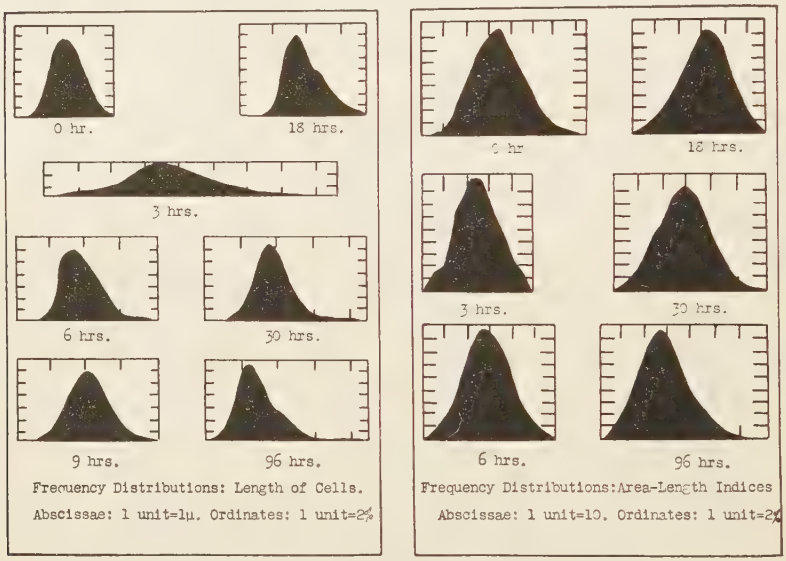

FIG. 26. FREQUENCY DISTRIBUTIONS OF CELLS ACCORDING TO LENGTH AND AREA-LENGTH INDEX, *B. coli.*

curve plotted logarithmically with regard to time, as well as to the number of cells. (Since cell counts were made only every three hours, this curve has been drawn in a rather free-hand fashion, but is probably fairly close to the true growth curve.) Again the marked increase in size during the period of active growth is apparent. The columns of cells have been arranged in the order of their area-length index, not in the order of their length, but it will be seen that as a matter of fact this method does roughly classify them according to their size. The longest cells in each column are

the cells with the lowest area-length index, that is the most slender, and the cells of greatest area-length index, at the top of the columns (i.e. those approaching spherical form), are also for the most part the shortest.

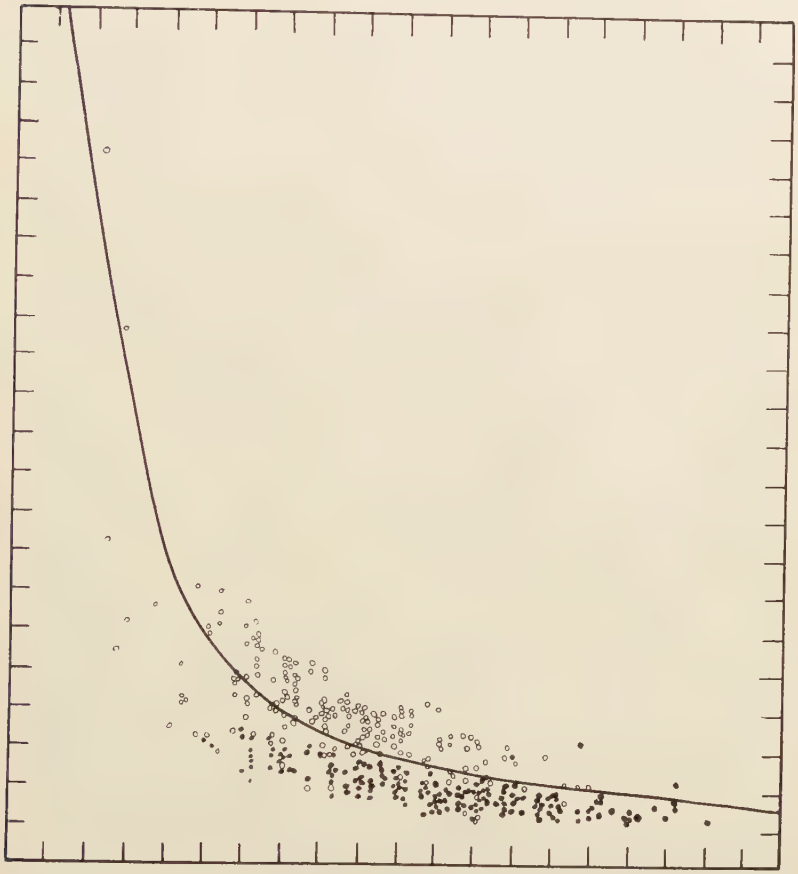

FIG. 27. CORRELATION GRAPH OF LENGTH OF CELLS AGAINST AREA-LENGTH INDEX, *B. coli*.

Ordinates, 1 unit = 3μ (Length). Abscissae, 1 unit = 3 (Area-length Index). Solid dots represent cells from eighteen-hour sample, hollow dots cells from three-hour sample.

Frequency distribution curves for the length of the cells are presented in Figure 26, together with curves for the distribution of

the cells according to their area-length indices. The curves for length show the increased variation in length during the period of active growth which was previously observed with *Bacillus megatherium.* There is, however, not nearly so pronounced a skewness in the curves and no tendency toward bimodality. The culture studied, however, was relatively heavily seeded, and it is probable that if further studies were made with this organism, with lightly seeded cultures, as has been done in the case of *Bacillus megatherium,* similar increased variation and a tendency toward bimodality in the distribution curves would be noted. The frequency distribution curves for area-length index of the cells show nothing very striking, save that during the period of active growth (at three hours) the range was somewhat restricted and the variation in form is somewhat less. The negative correlation between size and area-length index, then, is also evident in the distribution curves.

The relation between size and form of the cells may be more clearly seen in a correlation graph (Figure 27) in which the length of the cells is plotted against the area-length index. For this graph the data from the three-hour sample, as representative of the maximum growth phase; and from the eighteen-hour sample, as representative of the resting phase, have been chosen. A curve has been very roughly fitted to the data by simple inspection. This curve is clearly hyperbolic, the ascending limb approaching as a limit an area-length index of zero, i.e., a form in which the thickness is infinitely small in proportion to the length; the descending limb approaches as a limit a form in which the length equals the thickness, i.e., a sphere. Such a hyperbolic curve would result if the cells varied in length but remained constant in diameter. That such is not the case is apparent from an inspection of Figure 24; the three-hour cells are distinctly thicker than the others. But *proportionately* to length, the increase in diameter is not great, and consequently a hyperbolic curve results when length is plotted against form. It is interesting in this connection to note that Minoux has recently concluded from theoretical-mathematical considerations that bacterial cells must grow much less in diameter than they do in length.

* * *

Sufficient information has now been presented to warrant some speculation regarding the significance of these variations in the size and form of the cells of bacteria during the period of active growth. While at first glance the simple explanation of Clark and Ruehl that these variations are merely due to the growth of the cells preparatory to cell division would seem sufficient, it should be clear by now that such an explanation is quite inadequate. I have previously mentioned my belief that these changes in size, considered together with certain other facts, are an indication that during the period of active growth we have, with bacteria, to deal with a special cell type characteristic of this growth phase, different in internal constitution as well as in external form from the resting cells with which we are mostly familiar. These young cells are therefore in a sense analogous to the embryonic cells of a growing multicellular organism. Now if we look upon them as having actually the significance of such embryonic cells, there is opened up an attractive field of speculation from the standpoint of phylogeny, for such embryonic cells should tend to revert to an ancestral type. The fact that with most species of bacteria these embryonic cells are long and slender, tending towards a filamentous form, suggests the possibility that these are attempting to form mycelium; that the bacteria are actually descendents of higher fungi. Such an hypothesis is, however, pure speculation, hardly warranted by the data at hand.

More profit would seem to accrue from a consideration of the data from a physical standpoint. Since the size of the cells continually increases as long as the growth rate is accelerating, the synthesis of protoplasm during this phase proceeds more rapidly than does cell division; and conversely, since the cells become increasingly smaller when the growth rate becomes slower, cell division must in this period proceed more rapidly than does the synthesis of protoplasm. Since increase in size is accompanied by increased relative slenderness, i.e., by a greater tendency towards the cylindrical or filamentous form, and the subsequent decrease is accompanied by a greater and greater tendency towards the spherical form, it follows that during the period of accelerating growth there occurs an increased amount of surface in proportion to the volume of protoplasm for each cell, and that during the phase of negative

acceleration the surface-volume ratio is decreased. It should be pointed out, however, that while this is true as long as individual cells are concerned, the reverse condition obtains when we consider the total amount of protoplasm, a greater surface being presented by a mass divided into small particles even if these be spherical, than by the same mass divided into larger particles, even though these are filamentous. These considerations indicate that we may with advantage consider the variations of the cells both in size and form from the standpoint of surface phenomena.

"Among the forces which determine the forms of cells" says D'Arcy Thompson, "whether they be solitary or arranged in contact with one another, this force of surface tension is certainly of great, and is probably of paramount importance." With organisms as small as bacteria, where the surface in proportion to mass of protoplasm is so greatly increased, this force must be, if possible, of even greater importance than with larger cells; Thompson explains in this way the relatively slight variation in form encountered with bacteria as compared with higher organisms. While it would take us too far afield (and require a much more extensive knowledge of the subject than I possess), to discuss in all its details this relationship of surface tension to the size and form of the cells of bacteria, a word or two on the matter may well be interpolated here. For a more detailed discussion of the matter, the reader is referred to Thompson's work.

If bacterial cells were at all times homogeneous masses of protoplasm, their cells should at all times be spherical, as with the cocci in the resting phase. But since with all forms, including the cocci when actively growing, the cells are elongated, there must be present some axially disposed force, some polar distribution of substances (and consequently of energies) to give them this oval or cylindrical or filamentous form. It is known with higher organisms, and generally assumed with bacteria, that forms other than the spherical are maintained by a certain rigidity of the cell wall due to the deposition of solid substances there. But, of course, the cell must have assumed its form previous to the deposition of this substance; consequently something must have been opposed to the rounding effect of surface tension previous to the development of rigidity in the wall;

i.e., while a rigid cell wall may maintain a form other than the spherical, it cannot give rise to it. Frobisher suggests that with bacteria, since they elongate perceptibly without changing in diameter, the rigid cell wall, if it exists, may be open at the ends. The fact that the ends of cells where they are not in contact with contiguous cells are always rounded supports this view.

If the elongated form of the cells is due to some internal, axially disposed force, opposing the rounding effect of surface tension, then when placed in a medium of lowered surface tension, this force meeting less opposition should produce a more pronounced effect, and the cells should become even longer and more slender. This in fact is what occurs. Frobisher noted that the morphologic variations induced by adding surface tension depressants to the medium were comparatively slight, but that with low tensions (35-40 dynes) the cells became longer and more slender. He observed but slight variation because he was dealing with cells which had passed the maximum growth phase. In some as yet uncompleted observations* on the effect of sodium ricinoleate upon the size and form of the cells of the colon bacillus, the soap being added in excess to agar, I have found that whereas after twelve hours or so the cells were only somewhat longer and more slender than in control cultures, during the active growth phase (three to five hours) they were astonishingly elongated and filamentous in form, many cells extending over several oil immersion fields. On the other hand, when calcium chloride (which tends to raise the surface tension somewhat) is added to the medium, the cells are somewhat shorter and more oval in form than in the normal medium.

If it be granted then that the elongation of the cells (with corresponding increasing slenderness) is due to the effect of some internal force opposing the surface tension of the medium, then we must assume that this force is greater during the period of increasing

* These experiments are uncompleted and are not reported in detail here because of the technical difficulties involved. It is impossible to determine the surface tension of agar after it has jelled, and difficult to obtain clear morphological studies from liquid media. The colon bacillus becomes strongly clumped in the soap medium so that it is impossible to obtain accurate measures of growth by any method. The growth forms a slimy mass which does not stain readily and which is difficult to spread in thin films.

growth rate than during the resting phase, and that it becomes increasingly greater with increased acceleration of growth; that any external or internal factor which tends to speed up growth also tends to increase the magnitude of this force.

Clark and Ruehl noted that the large forms which they found during the active growth phase were much more intensely stained by the basic aniline dyes than were the "normal" forms found in later stages of growth, and I have observed the same thing, the intensity of staining being very striking. Eisenberg points out that the Gram-staining of bacteria is variable with the age of the culture, and that in very young cultures certain of the Gram-negative bacteria may retain the dye. Stearn and Stearn have shown that the reaction of bacteria to acid and basic dyes depends upon the iso-electric "zone" of the amphoteric protoplasm of which they are composed, the difference between Gram-positive and Gram-negative bacteria being due to this difference in their iso-electric points. With this information at hand we may generalize a bit and conclude that in our "embryonic" bacteria the iso-electric "zone" is further on the acid side of neutrality than is the case with the mature form, just as in very old cultures it is further on the basic side, Gram-positive organisms becoming Gram-negative, all organisms staining more faintly with basic dyes, and some cells finally becoming stainable with acid dyes like Congo red, as was pointed out in Chapter III.

If we accept the diffuse nucleus theory for bacteria, is it not plausible that this increased affinity for basic dyes is due to an increased proportion of nucleoproteins distributed through the protoplasm? And is this not analogous to the high nucleus-protoplasm ratio characteristic of the embryonic cells of higher organisms?

Sherman and Albus (1923), as has been previously mentioned, have found that young, actively growing cells of the colon bacillus are much more susceptible to various injurious agents (heat, cold, sodium chloride, phenol) than are older cultures. They also noted that such young cultures were not susceptible to acid agglutination, which may be correlated with the low iso-electric point of their protoplasm as indicated above. They believe that these observations indicate a physiological difference between growing cells and resting cells, that there is a "physiological youth" in bacteria analo-

gous to the physiological youth which has been demonstrated by Child in multicellular tissues, as regenerating pieces of planarians. It is noteworthy that Child also demonstrated this physiological youth by increased susceptibility to injurious agents.

These three correlated properties: Increased length and slenderness of the cells, indicating a greater magnitude of some axially disposed force opposing the surface tension of the medium; increased intensity of staining with basic dyes and decreased susceptibility to acid agglutination, indicating an iso-electric point of the protoplasm more on the acid side; and increased susceptibility to injurious agents, all serve to distinguish the young, actively growing cells from the resting cells, and justify our recognizing these long cells as a distinct morphologic type, as *embryonic* cells, characteristic of the growth phase of the culture.

CHAPTER VI

SOME OBSERVATIONS OF A DIPHTHEROID BACILLUS

It will be remembered that Clark and Ruehl observed that the diphtheria and diphtheria-like bacilli showed a decrease in size during the early stages of growth, followed by a gradual increase. Albert, on the other hand, claims that the diphtheria bacillus increases in size during the first four hours of growth, remains stationary for about twenty hours, and then gradually decreases. This, however, I cannot confirm, as I find that during the actual growth period they become smaller. This group therefore deserves some particular study, but is not well suited for the type of work here reported because most of the members of the group are very small and are grown with some difficulty on artificial media. I was fortunate, however, in finding a saprophytic organism which seems to possess the characters of the Corynebacteria, that is quite large and could also be grown fairly readily. It is a red chromogen and was isolated from lake water. Since this organism is apparently a new species it will be described in some detail.

In morphology it resembles the Corynebacteria in the possession of very prominent metachromatic granules, in pleomorphism which is very prominent in old cultures, in a tendency toward a palisade arrangement of the cells at some stages of growth, and in the fact that during the early stages of growth the cells are much smaller than they are later. The length of the cells on ordinary agar may be found in Table XX. In other media, particularly Loeffler's blood serum and dextrose agar, they may be much larger. The diameter does not vary so much as the length. The cells also vary considerably in form. They are generally cylindrical with rounded ends; when in pairs or chains the opposed ends are flattened. According to the medium and age of the culture they vary from short oval cells, sometimes almost spherical, to long filaments. In old cultures, cells of irregular shape appear. In young cultures, particularly on

dextrose agar, the cells are arranged in short chains; later these break up and the cells are singly distributed or clumped in the parallel formation characteristic of diphtheria bacilli. It is non-motile. With Gram's stain the reaction varies with the age of the culture. In relatively young cultures, under forty-eight hours, the organism is Gram-positive, but like the diphtheria bacillus not strongly so, being readily decolorized if the action of the alcohol is somewhat prolonged.

The granules within the cells give all the microchemical reactions of volutin; they disappear after treatment with boiling water, they stain vitally with weak solutions of methylene blue and of neutral red, they retain methylene blue after treatment with 1 per cent sulphuric acid, Bismarck brown, and Gram's iodine, and they stain violet or reddish with old solutions of methylene blue and with toluidine blue. They vary in size, number, shape, and position within the cell with the age of the culture and the nature of the medium. In agar slant cultures the granules are very small, but because of their intense staining quite distinct. In a twenty-four-hour culture the majority of the cells contain two granules, situated near the extremities of the cell; but some cells may contain a considerable number scattered throughout. During the period of active growth, however, the granules decrease in number, and after six hours on agar most of the cells have no granules, and those that do seldom have more than one, which is centrally located. In the involution forms which appear in the old agar cultures the metachromatic material is variously disposed, sometimes in large irregular masses, frequently as a long, narrow, twisted filament in the middle of the cell. In liquid media the granules appear much as on agar, save that the variations occur much more slowly. On Loeffler's blood serum the granules are very large, sometimes bulging the cell, and round. When the granules are quite large, as on this medium, they appear to stain more intensely at the periphery.

In media containing dextrose the granules more frequently occupy a position in the middle of the cell, and apparently divide preceding cell division. This was most marked in a "synthetic" medium composed of ammonium sulfate, dibasic potassium phosphate, magnesium sulphate, and dextrose. Here the cells were for

the most part oval in shape, and in a resting state exhibit but one large granule centrally located. From the various appearances presented by a series of cells from such a culture, it would appear that, preceding cell division, the granule first elongates in a direction at right angles to the axis of the cell, then splits in two along its own axis, after which the cell divides on a plane between the two granules, and each of the new cells is thus provided with one granule, at first situated at one pole, but later moving to the middle of the cell and again becoming rounded. This apparent participation of the metrachromatic granules in the process of cell division has been noted by Ernst and by Williams, who have suggested that the granules must therefore be of the nature of nuclei. The fact, however, that the division of the granules preceding cell division occurs only at times or in certain media, and only in those cells where the granules happen to be located at the point of division, would argue against their performing any essential function in the process; and it is not necessary to assume that they possess either the structure or the function of nuclei to explain the phenomenon, for it is clear that if they happen to lie in the plane of fission they will be acted upon by the forces which cause the cell to divide and must themselves be divided.

The most striking of the cultural characters was the pigment formation, which remained quite constant throughout the period (several years) that the organism was under observation. It was always a tint of grenadine red, usually grenadine pink (7d, Ridgway's Color Standards and Nomenclature). The pigment is extracted from the wet cells by ethyl and methyl alcohols, by acetone, and by 20 per cent sodium hydroxide, but not by ether, petroleum ether, toluene, xylene, or 10 per cent hydrochloric acid. From the sodium hydroxide solution it can be shaken out with chloroform, and acetone, only slightly with petroleum ether, and not at all with ether, toluene, or xylene. From the methyl alcohol solution somewhat diluted with water, it can be shaken out with petroleum ether, more readily with carbon disulfide. The growth on agar turns a bluish green when treated with sulphuric acid. The pigment is a lipochrome very similar to carotin.

On solid media the growth is smooth and glistening, somewhat

mucoid in consistency. In liquid media there is produced a diffuse turbidity; after some time a slight pellicle appears. Gelatine is rapidly liquefied, but egg, blood serum, and casein are not digested. No indol is formed. None of the common sugars are fermented. The organism grows best on slightly alkaline media and is sharply inhibited at pH 6.4. It seemed to grow equally well at temperatures between 30° and 37°.

Although various bacteriologists, notably Harris and Wade, have noted the occurrence of saprophytic chromogenic diphtheriod bacilli, which are common and widely distributed, these organisms have not been thoroughly studied. I have found in the literature but one producing a red pigment which has been adequately described and named. This was reported by Hoag as "Bacillus X", and named by Morse *B. hoagii,* which was changed to *Corynebacterium hoagii* by Eberson. The organism here described differs markedly from *C. hoagii,* however, in the unusually large size of the cells, the deeper color, the liquefaction of gelatine, and the failure to ferment dextrose and sucrose. While it grew very readily when first isolated on all sorts of media, after a few years subcultivation it gradually gave a scantier growth and finally died. Since the type culture is therefore not available, I hesitate to name it.

The rate of growth, length of cells, and number of metachromatic granules in the cells have been determined from a series of agar slant cultures inoculated from a forty-eight-hour old agar culture, following the technique described in Chapter III. Measurements of cell length were made from Congo red slides projected at a magnification of 3,000×. The granules were counted from slides stained by Ponder's method with toluidine blue; and for purposes of correlation between number of granules and size of cells the length of the cells was also determined from these latter slides by means of a camera lucida and dividers, the magnification being 1,500×.

The general character of the morphologic variations observed can be seen in Figure 30, which presents a series of camera lucida drawings from cultures of different ages. These were made from cultures on dextrose agar, in which medium the changes in the appearance of the cells are more pronounced than on ordinary agar which was used for quantitative study; but the cell variations are

of the same kind. The resting cells are large, with a faintly stained protoplasm and deeply stained granules. When growth commences they become much shorter, almost spherical in form, arranged in chains; the granules disappear, and the entire protoplasm becomes deeply stained.

The results of the various measurements are presented graphically in Figure 28, and in tabular form in Tables XIX, XX, and XXI. In Figure 28 the logarithms of the numbers of cells are plotted as a solid line, the average length of the cells as a dotted line, and the average number of granules per cell as a dot-and-dash line. The measurements of cell length show very pronounced minor fluctuations which must be due to experimental error, but the major tendency is clear. During the period of accelerating growth rate the size shows very little change; if anything, the cells increase somewhat. But as soon as the growth rate begins to slow up there occurs a very pronounced decrease in the size of the cells, which reach their minimum size at about the point of inflection between the growth phase and the resting phase. The return to the original size proceeds very slowly; the original size is attained only after the culture had been in the resting phase for some twenty-four hours. Since the growth curve was determined by cell counts rather than by plate counts, it is quite possible that the culture had entered the death phase before the original size was reached. While measurements were not continued longer than forty-eight hours, simple inspection of the slides shows that they continue to elongate, becoming long filaments after some days.

It will be seen at once that the variations in cell size with this organism are of quite different nature from those found with *B. megatherium* and the colon bacillus. It might be argued that the slight initial increase in size apparent during the period of accelerating growth is the same sort of a phenomenon as the pronounced increase noted with the other two organisms, and that the pronounced decrease during the period of negative acceleration is a peculiarity of the diphtheroid group. In favor of this hypothesis is the fact that the granules disappear and the protoplasm becomes rather deeply stained before there occurs any decrease in the size of the cells. But Clark and Ruehl failed to observe any initial increase in the size

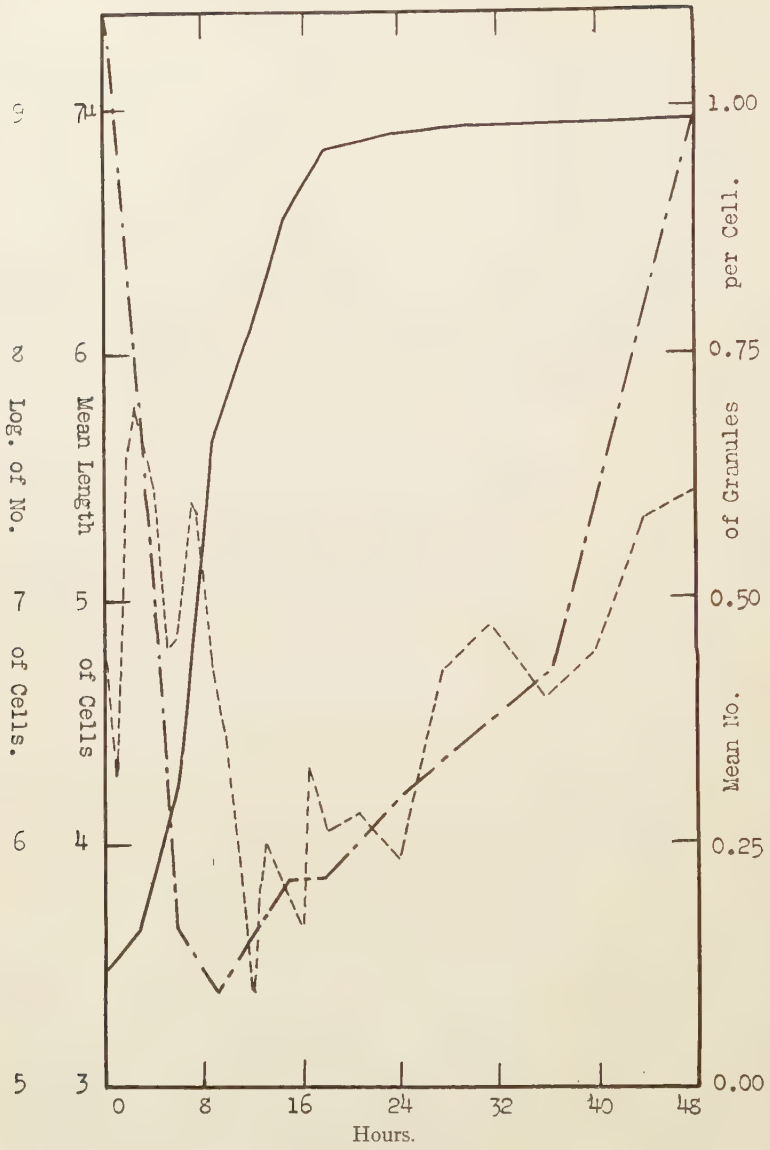

FIG. 28. GROWTH CURVE (SOLID LINE), LENGTH OF CELLS (DOTTED LINE), AND METACHROMATIC GRANULES PER CELL (DOT-AND-DASH LINE), DIPHTHEROID ORGANISM.

of the cells of the *Corynebacteria* which they observed. It is noteworthy that their curves also show a very slow increase to the original size of the cells as compared with the very rapid decrease to the original size observed in the other organisms studied. It is evident that more work will have to be done with this group before the changes in cell size can be clearly correlated with the phases of growth. From the curve here submitted it would seem that the small sized cells are more characteristic of the resting phase than of the growth phase, but from evidence to be submitted later it is suggested that this may be wrong.

That this diphtheriod organism behaves differently from the other bacteria studied with regard to its variations in cell size is still more apparent when studied from the standpoint of frequency distributions, illustrated in Figure 29. No such pronounced variation is apparent as was the case with *B. megatherium* and the colon bacillus; and the increased variation occurs not during the period of most active growth, but during the resting period. The frequency curves at six and twelve hours are practically symmetrical; all of the others show some skewness. It will be noted from the coefficients of variation in Table XX that, while this value shows considerable fluctuation, it is distinctly lower during the period from nine to twelve hours, i.e., during the stage of decreasing size of the cells, a period during which the growth rate is decreasing but is still relatively high. While the cells are increasing in size again during the resting phase, the coefficient of variation also increases. It is clear, then, that variability in size is correlated with the actual size of the cells, not necessarily with the rate of cell division. This is noteworthy because Taliaferro and Taliaferro have used the coefficient of variation as a measure of the rate of growth in trypanosomes, on the supposition that during active cell division there will be found more variation in size because of the presence in the population of cells which are mature, cells which have just divided, and all stages between; whereas when growth ceases, there being no further cell division, all of the cells will be much of a size. This of course will be true only if the cells show no other change in size than that preparatory to division. However true it may be for trypanosomes, it will not hold for bacteria. With the diphtheriod organism here

described the minimum coefficient of variation corresponds nearly with the maximum rate of cell division.

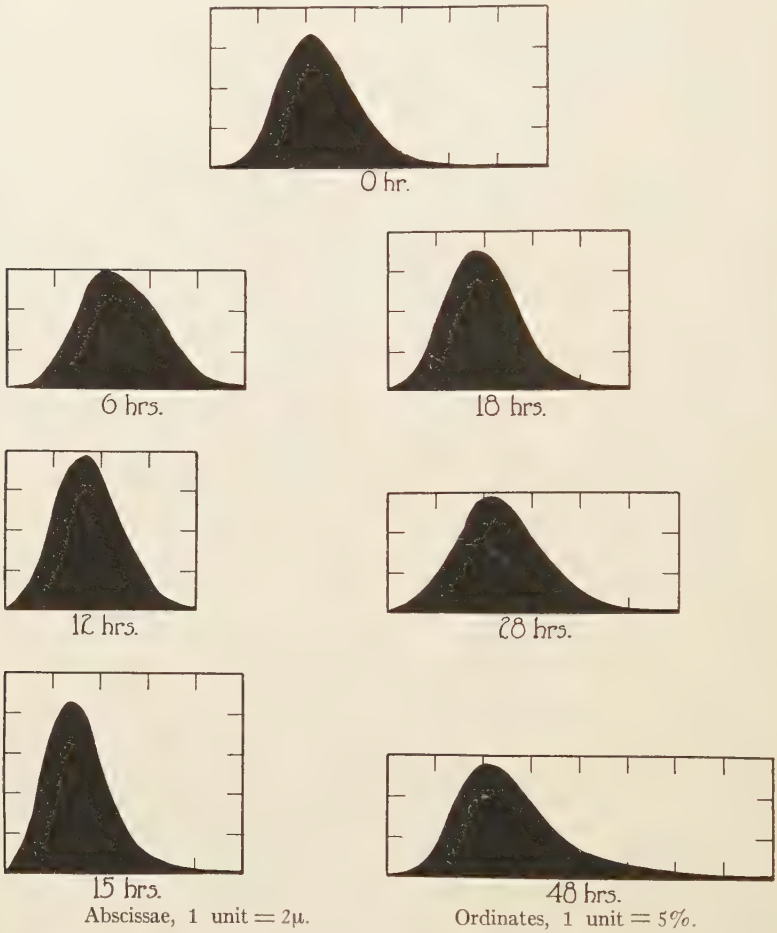

FIG. 29. FREQUENCY DISTRIBUTIONS OF CELLS ACCORDING TO LENGTH AT DIFFERENT STAGES OF GROWTH, DIPHTHEROID ORGANISM.

It has long been known that the metachromatic granules or volutin of microörganisms tends to disappear during active growth, and to reappear again after growth has ceased; in fact the generally accepted view that these granules represent some sort of reserve

food material is based upon this observation. Albert states that in the case of the diphtheria bacillus:

> Granules appear in cultures four to eight hours old. These are at first of small size. Granules attain their largest average size in cultures twelve to fifteen hours old. After this period the granules diminish in size and disappear. The percentage of bacteria which contain granules increases rapidly from the four-hour period when there are but a few granules to the twelve-hour period when 91 per cent contain granules. At the end of two days most of the bacilli have lost their granules.

The curve for the mean number of granules per cell in Figure 28 shows that the granules rapidly disappeared with active growth, the minimum number being found at nine hours (when there was only one granule to every ten cells), which corresponds approximately with the point of maximum growth. But the granules came back very slowly, not nearly so rapidly as was noted by Albert with the diphtheria bacillus. This is probably due to a difference in the nature of the organisms, though perhaps in part to the fact that he was using a much more favorable medium and probably much more heavily seeded than my cultures.

The mere number of granules is not a very good measure of the amount of volutin in the cells, since the granules also vary greatly in size, in general being smallest when they are fewest, and gradually increasing with age of the culture until they nearly completely fill the cell. I have not noted their disappearance during the late stages of growth as has been described by Albert; on the contrary, they become more and more prominent, and very irregular in form in the large filamentous and irregular cells found in old cultures.

It will be noted in Figure 28 that the curves for cell length and for number of granules per cell follow the same general course, the approximation being greater during the later stages of growth than at the beginning, since the granules decreased at once, whereas the cells did not begin to shorten until after some hours. There is apparent then a degree of correlation between the size of the cells and the number of granules which they contain. I have calculated the coefficients of this correlation for the various samples in which the granules were counted (100 cells were counted and

measured), which are given in Table XXI. The coefficient is high when the granules are numerous, low when they are scarce. This is probably a spurious correlation; the two values happen to move together during part of the growth cycle, but are not actually related.

While one cannot clearly establish the relationship between the morphologic variations of this organisms and the phases of growth from the data at hand, it can be readily demonstrated that these variations are dependent upon the rate of growth by observing the effect of varying the size of the seeding and the concentration of nutrients in the medium. This has not been done quantitatively, but the drawings in Figure 30 show the effect. These were made from cultures on dextrose agar (dextrose 5 per cent, peptone 1 per cent) inoculated from a twenty-four-hour old culture on the same medium, the cells of which are illustrated in the drawing marked 0 hour. Three series were run in parallel. The two marked "Heavy inoculum" were seeded with the same number of cells; that marked "Light inoculum" received one-tenth as many. The two marked "Full strength medium" were inoculated onto agar slants having the composition given above; the one marked "Dilute medium" was planted onto agar slants having just one-fourth as much dextrose and peptone. It will be seen that in the dilute medium the morphologic variations were not so pronounced as in the full strength, and that with a light seeding there was more variation than with the larger inoculum. Those conditions which lead to a high growth rate, viz., small seeding and large concentration of nutrients, cause a development of more of the small, intensely stained, granule-free cells and their persistence for a longer period of time. This being the case, it is probable that these small cells are truly to be considered the form characteristic of the growth period rather than the resting phase, that they are the embryonic forms of this type of organism, and the failure of the curves to correlate in phase in Figure 28 is really due to some experimental error.

It is rather striking in these drawings that the small forms become almost spherical and are arranged in chains; this is especially evident in the light inoculum, full strength medium. At ten and fifteen hours the cells would hardly be recognized as being related

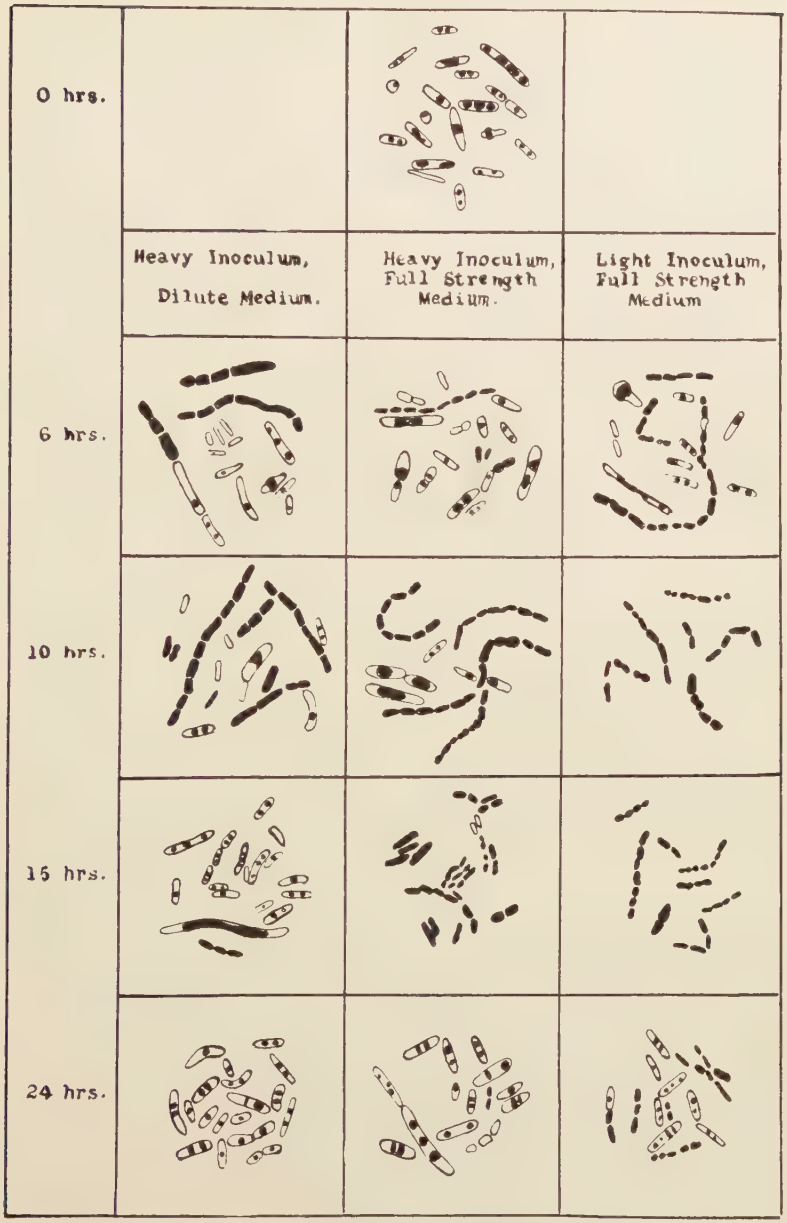

FIG. 30. INFLUENCE OF SIZE OF SEEDING AND CONCENTRATION OF NUTRIENTS ON MORPHOLOGIC VARIATIONS OF A DIPHTHEROID ORGANISM.

to those used for seeding; they look like streptococci. This is rather interesting because various authors, particularly Mellon, have suggested that the diphtheroids and the streptococci are closely related. If such a relationship exists, these observations might be construed to indicate that the diphtheroids have developed from the streptococci and during their embryonic phase revert to the ancestral type. At any rate it would seem likely that the so-called mutations of diphtheroids to streptococci as described, among others, by Mellon, may be the result of merely comparing different phases of growth, a culture on one medium being in the resting phase when on another it may still be actively growing.

CHAPTER VII

A NOTE ON SPORE FORMATION

It is frequently stated that bacteria form spores when conditions become unfavorable for growth. This is, however, only true in a narrow sense. Certain factors which tend to restrict the growth rate also tend to delay rather than to accelerate spore formation. Thus Migula has shown that spore formation is less rapid and complete on either side of the optimum temperature for growth. It is well known, however, that bacteria do not form spores until the culture has practically stopped growing; that spores are structures peculiar to the resting cells rather than to the actively growing cells. There is, however, as far as I can learn, no very definite evidence in the literature concerning the actual relationships of spore formation to phases of the growth curve.

This, however, is apparent in the data obtained on the growth of *Bacillus megatherium,* presented in Chapter IV. During the course of the investigation of the sizes of the cells in the first culture reported in that chapter, the number of free spores was incidentally determined, and is recorded in Table VI. The relationship of spore formation to the rate of growth in the culture is indicated in Figure 31, where the logarithms of the number of vegetative cells and the number of free spores are plotted on the same scale (sporangia being counted as vegetative cells, with only free spores being considered as spores). It will be noted from this graph that spore formation commenced practically at the point of inflection between the logarithmic growth phase and the resting phase; that after spore formation commenced it proceeded at a practically constant rate for some time, the logarithms of the number of spores falling on a straight line; but that spore formation also began to slow up after some hours, the rate continually decreasing during the period of observation. Not all of the cells, therefore, developed into spores, at least within the period of time that the culture was studied.

Since spore formation is a characteristic of the resting phase, the rate of spore formation should be influenced by those factors

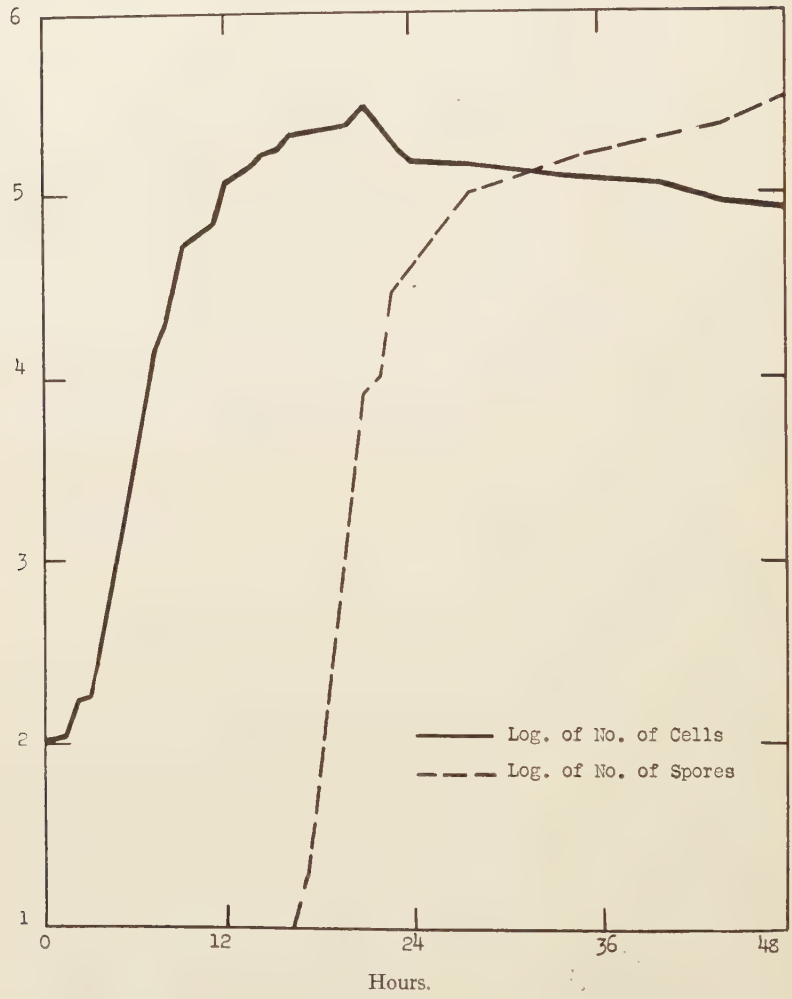

FIG. 31. RELATION OF RATE OF SPORE FORMATION TO RATE OF GROWTH IN *B. megatherium.*

which also tend to influence the rate of growth and the form of the growth curve. Migula has determined that spore formation

seems to depend upon the density of the population in the culture. He states that spore formation can be indefinitely postponed if the culture is transferred to a new medium at a period preceding the appearance of spores. It seems to begin after a definite number of cell divisions have taken place, spore formation appearing at a definite concentration of cells, regardless of the initial size of the seeding for a given volume of medium, and begins always when the medium is no longer suitable for vegetative multiplication, that is when growth has ceased. Migula also found that spore formation could not be delayed by the addition of nutrient to the medium in concentrated form (dry peptone and meat extract), but, on the other hand, by adding distilled water spore formation was postponed. He concluded, therefore, that spore formation was not due to an exhaustion of the medium; that it depended entirely upon the concentration of the cells, and was probably the result of an accumulation of products of metabolism of the bacteria.

In order to determine more clearly this relationship between rate of growth and rate of spore formation, the following experiment was performed: A culture of *Bacillus cohaerens* some days old, in which there were practically no vegetative cells, was inoculated on to four sets of agar slants, two of normal agar, the other two containing one-fourth as much peptone and meat extract. One set of each kind was inoculated with a very heavy suspension, and the other with the same suspension diluted 1-50. At regular intervals samples were removed and the percentage of free spores among 200 cells was determined from each sample. The results are given in Table XXII, and are presented graphically in Figure 32.

It will be noted from this graph that spore formation proceeded more rapidly in the media of lower nutrient value than in the full strength media, regardless of the size of seeding, and that it proceeded more rapidly in the heavily seeded cultures than in the lightly seeded ones, though the difference here was not so pronounced as was the effect produced by varying concentration of nutrients. In the heavily seeded dilute medium, spores never entirely disappeared, new spores starting to form before all of those introduced had germinated. It would appear, therefore, that the rate of spore formation is determined not by the concentration of cells alone, but rather

by the density of the population in relation to the concentration of foodstuff in the medium. It appears earlier in the heavily seeded cultures and in the more dilute media, because those cultures do not have so long a period of vegetative reproduction; that is, because the resting phase of the culture appears earlier. This is quite con-

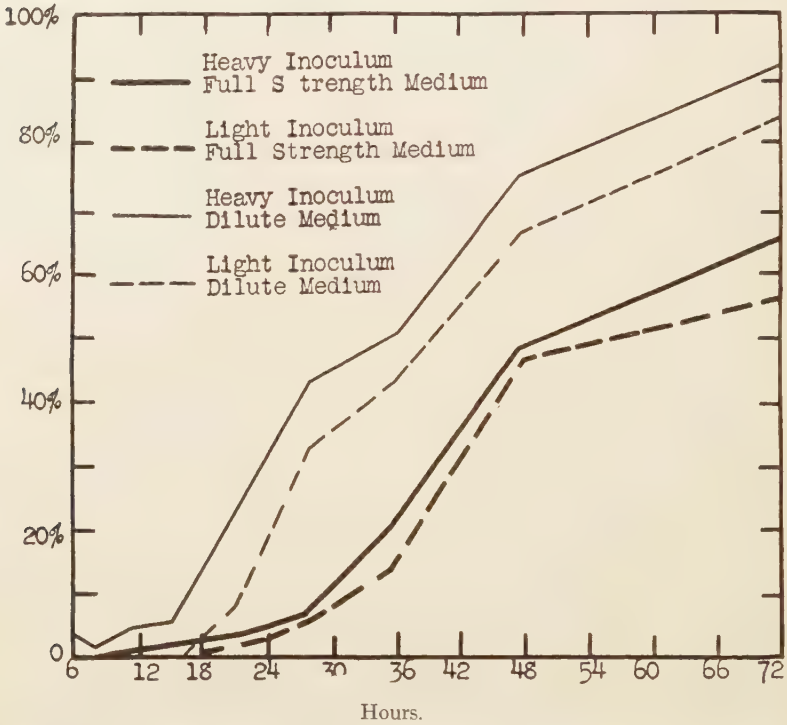

FIG. 32. INFLUENCE OF SIZE OF SEEDING AND CONCENTRATION OF NUTRIENTS ON RATE OF SPORE FORMATION IN *B. cohaerens*.

trary to the results obtained by Migula, and I cannot help but believe that his observations were in error.

* * *

If we are to consider the large, deeply stained cells free of granules as comparable with the embryonic cells of a growing plant or animal, as has been proposed in Chapter V, then conversely we must look upon those cells with which we are most familiar, the

cells characteristic of the resting phase of the culture, as being analogous to the differentiated cells which occur in the mature plant or animal. Minot states that differentiation consists essentially in this, that something new appears within the cell. I submit that the metachromatic granules observed in diphtheroid bacteria, the unstainable granules previously described in *Bacillus megatherium*, the spores which are found in many species of bacteria, are something new which appears within the protoplasm analogous to the various granules, secretory or reserve material, or of unknown function, which serve to distinguish the differentiated cells of a multicellular organism from their embryonic prototypes.

It has been shown in Chapter VI that the metachromatic granules begin to reappear with a decrease in the growth rate, and are most persistent in those cultures (heavily seeded, or dilute medium) which have the lowest growth rate; which I have just shown is also true of spores. Those factors then which tend to produce a short growth phase of low rate tend to give a maximal development of those characters which distinguish the mature or differentiated cells, as conversely they tend to give a minimal development of the embryonic types.

CHAPTER VIII

MORPHOLOGIC VARIATIONS OF THE CHOLERA VIBRIO

The organism of cholera has long been a favorite for studies of pleomorphism. Many bacteriologists, among them both pleomorphists and monomorphists, have found that this organism is more prone to morphologic variation than many other forms; that cultures of the same origin on different media, and cultures of different origin on the same media, which may be identical in other characters, may be widely different in the form of their cells. Short and long rods, "comma" forms, spiral filaments, large and small spherical cells, as well as budding and branching, bulging and irregular cell forms, have all been described over and over again. Most of these observations have been made on unusual media, though even in cultures on standard beef extract agar considerable variation is encountered. These variants from the typical vibrio form have been looked upon by some authors as involution forms, that is cells which have suffered an injury or are dead; by others, as unusual reproductive cells (for example, gametocytes by Almquist and Enderlein); and by still others simply as the result of the action of physical or chemical forces, particularly osmotic pressure, on the cells, implying neither necessarily an injury nor a change in the mode of reproduction (for example, as plasmoptysis by A. Fischer). For complete literature on the subject, the reader is referred to Löhnis' monograph on "Life Cycles of Bacteria."

It was thought desirable, therefore, to extend these quantitative studies to include this organism, and particularly to see if something could be accomplished in the way of applying statistical methods to variations in the form of the cells rather than their size. It will be remembered that with the colon bacillus it was found that the variations in form could be fairly readily expressed by the area-length index (the area of the projected image divided by the length squared), this measure serving to classify the cells into long fila-

mentous types on the one hand, and spherical cells on the other. But it was pointed out that this measure only suffices as long as the cells are symmetrical with regard to both axes. Cells which show terminal or lateral or central bulgings, cells which are branched, wedge-shaped cells, and such unusual types, are not classified by this measure in regard to their asymmetry; they are merely placed in the series according to whether their general form approaches the filamentous or the spherical. Such asymmetrical cells were found to be quite numerous with the cholera organism studied. Nevertheless this method of studying variations in form was carried through with the hope that, regardless of the asymmetry of some of the cells, it would serve to classify them, although roughly, at least to an extent which would give some idea as to the nature of the variations which are occurring.

A five-day-old culture was inoculated onto agar slants, from which samples were removed at regular intervals for observations. Photomicrographs were prepared and reprojected to give a final magnification of 21,000×, the cells being traced on to cards and measured as described in Chapter III.

The general character of the morphologic variations of this organism are clearly indicated in Figure 33. This figure has been prepared by plotting the logarithms of the number of cells against the logarithms of the hours of growth. Since the growth curve has been plotted from cell counts made by the direct microscopic method, it does not show the true rate of death, as death of the cells is not registered in these counts until they have autolysed and disappeared. I have indicated by a dotted line on this graph what I believe was approximately the true death curve as it would have been if plate counts had been made. This estimate may be justified by data to be presented later. Superimposed upon this curve columns of cells have been erected in the order of their area-length indices (those with the lowest index being at the bottom), choosing each tenth cell from the samples of 200 which were measured. The way in which the cells are classified by this value is apparent from inspection of the figure. The longest and most slender cells occur at the bottom, the cells becoming shorter and plumper as we approach the top of the column, those cells at the very top being

practically spherical. It will be noted also from this figure that the asymmetrical cells are not classified; that is, cells with terminal bulgings, for instance, may be found at almost any position in the column, their position being determined by their relative plumpness or thinness, rather than by their asymmetry.

It will be noted that during the lag phase practically no variation in the cells occurred. The cells used for inoculation consisted in part of typical vibrio forms, and in part of small oval and coccoid cells with some irregular budding and branching cells. With the

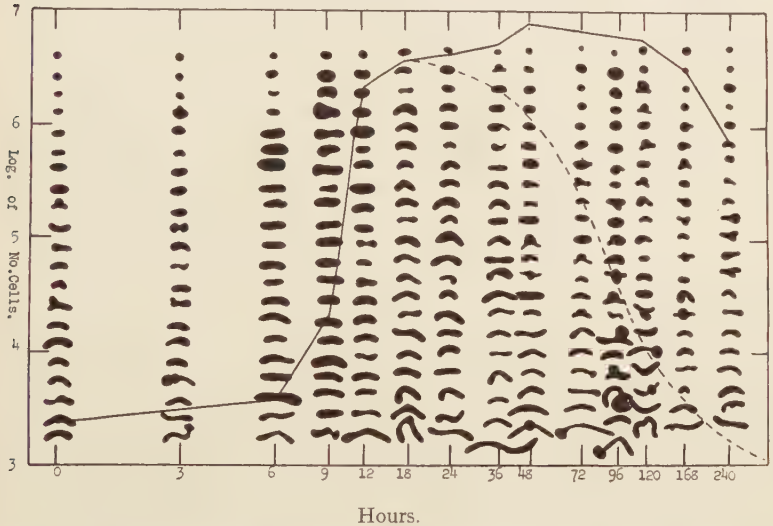

FIG. 33. REPRESENTATIVE CELLS FROM A CULTURE OF THE CHOLERA VIBRIO AT DIFFERENT STAGES OF GROWTH.

initiation of growth at six hours a definite alteration in form occurred, the majority of the cells being larger and relatively straight. These forms persisted during the period of active growth, but with the cessation of growth at twelve hours the cells became more slender and more curved, and during the resting period these typical vibrio forms were predominant. Beginning at thirty-six hours there appeared more and more irregular cell forms of the type which I have designated asymmetrical. Without any measurements, then, one can clearly distinguish in this graph the occurrence of three distinct types

of cells characteristic of the growth phase, the resting phase, and the death phase, respectively; the first being large, relatively plump, and straight, the second being more slender and markedly curved, the third being characterized by asymmetry of the cells.

The variations in size of the cells were relatively slight as compared with the variations noted previously in *Bacillus megatherium* and the colon bacillus. The mean length of the cells is given in Table XXV. There was a slight increase beginning at six hours, that is at the end of the lag period, which persisted to practically the end of the resting period (forty-eight hours), after which the cells showed a considerable decrease in length. Clark and Ruehl, however, found that the cholera vibrio showed an increase in length of cells of practically the same degree as that noted with the other organisms that they studied, and it is quite possible that in my observations the change in the size of cells is not as great as is usually the case. No studies of frequency distribution of the cells with regard to their size have been made with this organism.

More significant results were obtained from the study of the variations in form by means of the area-length index. These are given in Table XXIV. No pronounced variation occurred until after nine hours, corresponding approximately with the point of inflection between positive and negative acceleration in growth rate. After this period the area-length index decreased considerably, reaching its minimum at eighteen hours, which corresponds with the point of inflection between the growth phase and the resting phase, after which it again increased, remaining fairly constant at a high level during the death phase. Slenderness of the cells is, then, a characteristic of the resting phase, and plumpness a characteristic of the death phase. When plump cells are used for seeding, the latter half of the growth phase is occupied by a progressive increase in their slenderness.

While the area-length index serves to measure the cells with regard to their relative slenderness or plumpness, it does not take care of another variation in form characteristic of the cholera vibrio, namely the degree of curvature of the cells. This, however, can be simply determined by measuring the length of the cells (by means of dividers) along their major axis (L_1) and the shortest

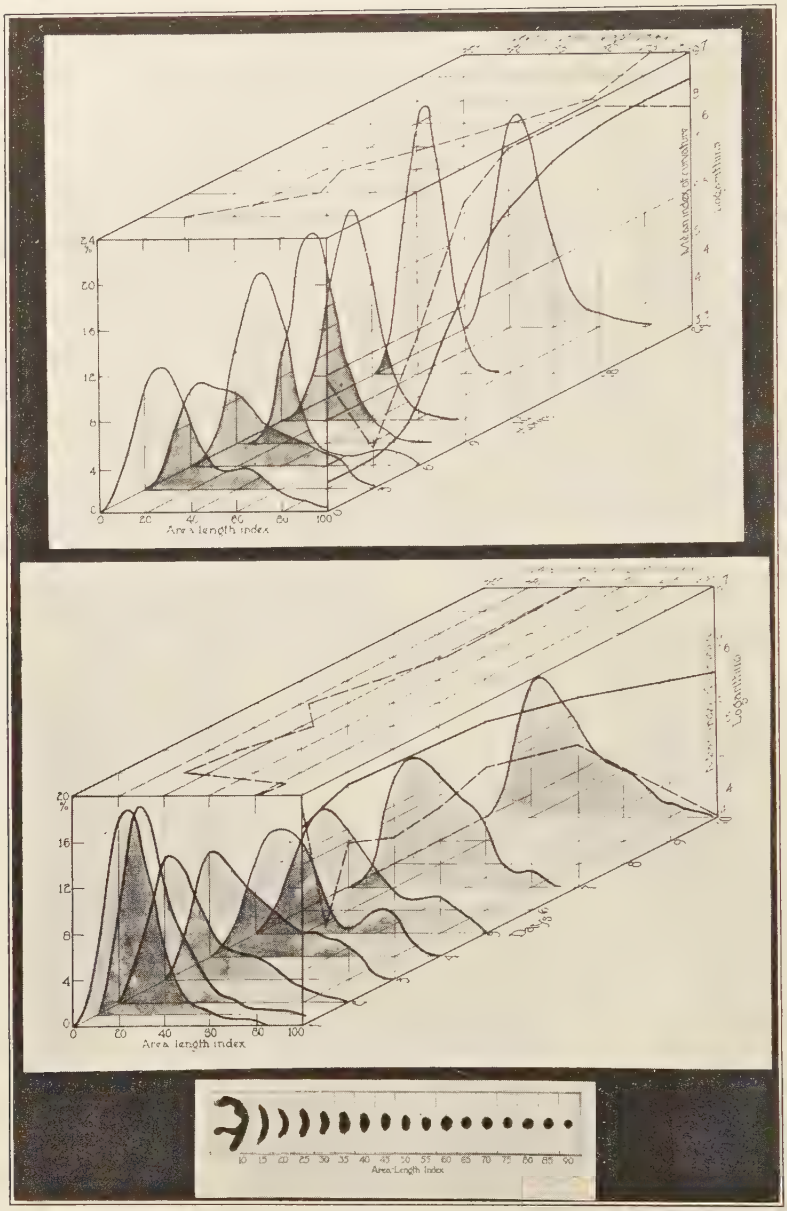

PLATE II. CORRELATION OF MORPHOLOGIC VARIATIONS WITH THE RATE OF GROWTH IN A CULTURE OF THE CHOLERA VIBRIO.

From "A Statistical Study of the Form and Growth of the Cholera Vibrio." Through the courtesy of the Journal of Infectious Diseases. (1925, **37**, 75).

distance between their tips (L_2). The proportion between the difference of these two values and the true length of the cells $\frac{L_1 - L_2}{L_1}$ serves as an abstract measure of the variation in curvature. The mean values of this index of curvature are presented in Table XXV. It will be seen that with the initiation of growth at six hours this value began to increase, reaching its maximum at twelve hours, approximately at the end of growth, and then progressively decreasing again to a minimum at the end of the period of observation, at ten days. That the degree of curvature is correlated with the rate of growth is quite apparent by an inspection of Plate 2. Particularly during the first twenty-four hours the curve for index of curvature is practically parallel with the growth curve. The maximum is maintained during the resting phase. Since the cells increase in curvature as they decrease in area-length index, that is as they become more slender, it seems probable that there would be found a correlation between the degree of slenderness and the degree of curvature, and this seems all the more apparent in the composite photographs given in Plate 2. When, however, the index of curvature was plotted against the area-length index with cells from the nine hour, eighteen hour, and thirty-six hour samples, no such correlation could be established. That is, while the period of greatest curvature corresponds with the period of greatest slenderness of the cells, it is not necessarily the most slender cells which are the most curved.

The frequency distribution of the cells with regard to their area-length indices is presented in Plate 2, where an attempt has been made to show in one illustration nearly all the morphologic variations of this organism. The frequency distribution curves have been erected in succession behind each other in isometric perspective. On the upper face of the parallelopiped containing them the mean area-length index has been plotted. On the right face the rate of growth is shown by plotting the logarithms of the number of cells. On this face there is also given the mean index of curvature. The significance of the area-length index is indicated in the small figure at the bottom of the plate, which presents composite photographs of all of the cells from the five-hour sample arranged

in classes according to their area-length indices. The values given are the maxima for the class, thirty for instance indicating all of the cells with area-length indices from 25.1 to 30.0. This shows well the variation from long, slender, curved cells, on the one hand, to small, spherical cells on the other.

It will be noted from the frequency distribution curves that the cells introduced into the medium apparently consisted of a large group of relatively slender cells distributed about one mode, and a small group of more oval cells distributed about another. As growth proceeded the frequency distribution curves exhibit a regular change in form. The minor mode is gradually absorbed and disappears; the range becomes restricted, the mode higher; and at eighteen hours we have a tall, symmetrical curve. With the beginning of the death phase the frequency curves again return to the form exhibited by the cells used for seeding; the base becomes extended, the mode lower; and there appears a new minor mode of oval cells, which reaches its maximum at four days, and then again disappears, the final curve at ten days being simply a much skewed curve with a broad base.

These variations in the frequency distribution are reflected in the coefficients of variability of the area-length index, presented in Table XXIV. This value showed considerable fluctuation, but was distinctly lower during the period of active growth from nine to eighteen hours, and increased again with the cessation of growth to reach its maximum at four days. Since the area-length index does not classify the asymmetrical cells, an attempt has been made to follow these roughly by simple inspection of the drawings, and the results are given in Table XXV. It will be seen that the percentage of asymmetrical cells steadily decreased during growth, the number remaining low until the beginning of the death phase at forty-eight hours, when they again increased, reaching a maximum at four days, after which there was again a slight decrease in number.

With all of these measurements at hand we are now in a position to say something more precise concerning the three types of cells previously mentioned. During the initial period of dormancy the cells showed no pronounced variation. With positive accelera-

tion, in growth rate there was an increase in size, a decrease in index of curvature, and only a very slight decrease in area-length index. The embryonic cells are therefore large, plump, and straight. With negative acceleration in growth there occurs a steady increase in the index of curvature and a decrease in the area-length index, with but little change in length. The maximum index of curvature and the minimum area-length index are found during the resting period. Therefore the mature cells are slender and curved, the typical vibrio form. The death phase is characterized particularly by increased variation in form, as indicated both by the coefficient of variability of the area-length index, and by the percentage of asymmetrical cells; further by the bimodality of the distribution curves indicating a tendency to break up into two groups of cells, one elongated, the other oval or spherical. These asymmetrical and coccoid cells are therefore to be looked upon as the senescent forms.

The asymmetrical cells found in the death phase will be recognized as those types which have so frequently been described as unusual reproductive cells, forming either buds or conidia or sexual cells. It would seem at first glance that probably the small oval or spherical cells have been derived from the longer, more slender cells by such a process of budding, and this impression is strengthened by the bimodality of the frequency distribution curves at this time, for if the small, oval cells were produced simply by a contraction of the longer cells, one would expect this process to be continuous, that is to find all transitional stages between the long, slender and the small, spherical types. If, however, a small portion of the cell were constricted off, it would naturally be more oval or spherical than the parent cell from which it was derived, and there would be lacking such transition cells, and therefore a bimodal frequency curve should result. It is curious that this bimodality in the frequency curves disappears with continued observation, and that during the latter stages of growth the curves tend again to become more symmetrical. This might be explained on the basis that the small, oval, or spherical types are dead, that they ultimately disintegrate, leaving behind only the more resistant slender types. Simple observation, however, without any measurements, seems to indicate that this is not the case; that with continued age of the culture the

tendency is for the cells to become smaller and smaller and more and more spherical, cultures a month or more old, for instance, appearing much like cultures of staphylococcus.

CHAPTER IX

THE SENESCENT FORMS OF THE COLON BACILLUS

The occurrence of irregular cell types which I have designated as asymmetrical in the preceding chapter seems to be clearly correlated with death. Now these cells are just those forms which have received the greatest attention from the exponents of life cycles in bacteria, and just those types which have been most the subject of controversy between the monomorphists and pleomorphists; the former, by designating them involution forms, imply an abnormality due to actual or approaching death; the latter believe them to be reproductive cells of one type or another. I do not believe that any data have as yet been brought forward to definitely settle this problem, for it may well be that those factors which lead to the death of some of the cells in a culture may react upon others to cause them to develop into one or another type of resting cell or reproductive body; but their occurrence only in the death phase is strongly presumptive evidence of the orthodox view that they are involution forms, and places squarely upon the pleomorphists the burden of proving that they are otherwise. By naming these irregular types "senescent" forms I express my opinion that the monomorphistic teaching regarding them is correct. But I prefer this term to "involution form" because it clearly expresses the analogy between them and the cell changes which occur in old age in multicellular organisms, and because it does not so much imply that they are already actually dead.

The observations recorded in preceding chapters have for the most part not been continued long enough to include more than the very beginnings of the death phase. In none of them have plate counts been made, so that it is not possible to determine the true rate of death. Moreover, the measures of morphologic variation used do not reveal except roughly the changes in the cells which seem to be most characteristic of the death phase. It was thought desirable therefore to continue these studies with some ob-

servations of the degree of variation in form of the cells during the death phase; to devise a measure of this variation which would consider all of the changes in form that occur; to include in the investigation the decrease in number of living cells by plate counts, as well as the decrease in total number of cells by microscopic counts; and to carry on the study in a number of different media with the idea of thus varying the death rate and so determining more conclusively whether or not the variations in cell form were directly correlated with the rate of death. Very little work has been published upon factors influencing the natural death rate in cultures, and since it could not be predicted that this would vary with the size of seeding and the concentration of nutrients, as does the growth rate, it was thought that more would be accomplished by using media of different composition, such as has been done by most of the authors who have described unusual cell forms, in an attempt to obtain the same types of morphologic variation as they have observed.

With these aims in mind the following experiment was performed. Five lots of agar were prepared, each having as nutrient 2 per cent of peptone. These proved to be neutral (pH 7.4). One lot was kept as such, another was made acid (pH 4.5) by the addition of HCl, a third alkaline (pH 9.2) by the addition of NaOH. The fourth had added to it 5 per cent of NaCl; the fifth, 3 per cent of $CaCl^2$. These were inoculated with a stock culture of the colon bacillus, the same strain as was used for the observations recorded in Chapter V. It was not possible to make all of these observations simultaneously, but conditions were kept as much alike as possible; the media were all made from the same lot of peptone, the cultures were inoculated from twenty-four-hour agar slants suspended in water to about the same density. The tubes were incubated at 37° in a moist chamber. Samples were removed after one, two, three, five, seven, ten, fourteen, nineteen, and twenty-five days (the final sample of the NaCl series was removed on the twentieth day, listed in Table XXVII as the nineteenth day). Five tubes were removed for sampling, the growth of each suspended in 5 c.c. of sterile water, and the contents of the five tubes were then pooled. From this suspension a 1 c.c. sample was removed and serially

diluted in sterile tap water for plating. Plates were made for the most part in triplicate, though on a few occasions five plates were poured for each dilution. The counts recorded are the mean of all plates of the same dilution, that dilution being chosen which gave the nearest to one hundred colonies per plate. Another 1 c.c. sample was mixed with Congo red solution, and slides were prepared for counting and photographing preparatory to measuring their size and form. The photographs were projected to give a final magnification of 19,000×; the image of the cells was traced on cards as previously used for the cholera vibrio. In addition to the direct microscopic counts the proportion of cells stained by Congo red was also recorded, and this yielded information of considerable significance.

The measurement of the rate of death in a culture, and the determination of the limits of the death phase present some difficulties. The death phase has generally been defined as beginning at the point where the number of living cells determined by plate counts begins to decrease, but undoubtedly the factor which causes the cells to die, whatever it may be, actually begins to act upon them earlier than this, at least at the point of inflection in the growth curve from positive to negative acceleration in rate of growth, and possibly earlier. This has been claimed by Wilson, who finds at all stages of growth a discrepancy between the microscopic count and the plate count, which can only be explained by the fact that some of the cells are dying, while the majority of them are still growing. By the time the culture has reached the beginning of the death phase, as defined above, there must be already a considerable number of dead cells present, and this is clearly indicated in my own observations here reported by the appearance in every case of a considerable number of cells stained by Congo red before there had occurred any decrease in the plate counts. On the other hand, when the number of living cells shows a decrease, and the culture may definitely be said to have entered the death phase, some of the cells are still multiplying, as indicated by the increase in microscopic counts, which generally continues for some time longer.

In these experiments we have three sets of data from which to compute the death rate; the plate counts which indicate the decrease

in actual number of living cells, the microscopic counts which indicate not only the death of the cells but also their complete autolysis and disappearance, and the proportion of cells stained by Congo red. These values are given in Table XXVI. The property of staining by Congo red, as was described in Chapter III, indicates not only the death of the cells but also that they have undergone a certain degree of autolysis. This being the case, as death proceeds the plate counts should show a marked decrease before the microscopic counts, and the proportion of stained cells should also increase at a slower rate than the plate counts decrease. Such was found to be the case. In order to show these three quantities graphically, in a form in which they might be directly comparable, the curves have been plotted in the following manner: The percentage of decrease from the maximum plate count was computed for the first day in which a decrease was noted, and to this was added day by day the further decreases in number of cells as a percentage of the maximum number attained in the culture. Thus we obtain a cumulative percentile curve which is directly comparable to the curve for the percentage of stainable cells, which of course is also cumulative. In a similar manner a curve for cumulative percentile decrease in the number of cells in microscopic counts was plotted. These three curves are given side by side for each of the five cultures in Figure 36. It will be seen that in every case the curve for percentage increase in stained cells (dotted line) occupies a position between the curve for percentage decrease in viable cells (solid line) and the percentage decrease in microscopic counts (dot and dash line).

In most cases the decrease in microscopic counts was not marked even at the end of twenty-five days. Their curves are therefore not completed and not easily compared with the curves for plate counts. The curves for stainable cells, however, are nearly complete in most cases, and the distance between these curves and the curves for plate counts then serves as a fairly good measure of the rate of autolysis in the culture. This measure can be best taken on the 50 percentile line, and is indicated in the graph, the figures given being the number of days which have elapsed from the time that half of the cells were dead, as determined by the plate counts,

and the time that half of the cells remaining in the culture have become stained by Congo red.

The rate of autolysis so determined proved to be lowest in the alkaline culture (17 days), the other cultures in order of increasing autolysis rate being the neutral one (13.25 days), the salt medium (6.25 days), the acid agar (4.25 days), and the medium containing calcium chloride (4 days).

The highest rate of death was shown by the salt culture, the mean daily decrease in the plate counts being 21.8 per cent; in the alkaline medium it was 16.5 per cent, in the acid one 16.4 per cent, in the calcium chloride agar 11.9 per cent, and in the neutral culture 9.7 per cent. The period of maximum death rate occurred in the neutral agar between the second and third days, the decrease per day being 60.2 per cent; in the acid medium between the fifth and seventh days, 37.7 per cent; in the medium with sodium chloride, fifth to seventh days, 32.2 per cent; in the calcium chloride agar, second to third days, 26.8 per cent; and in the alkaline culture, tenth to fourteenth day, 23.3 per cent. The order of the cultures with regard to rate of autolysis does not agree with either of the above, and the rate of autolysis is therefore independent of the death rate.

The medium most favorable to growth, as measured by the maximum yield (plate counts), was the acid agar; the others, in descending order being the alkaline, the neutral, and those containing salt and calcium chloride. The death phase began earliest in the neutral and alkaline media, both showing the maximum plate counts on the second day; in the salt agar and calcium chloride medium on the third day; and in the acid agar on the fifth day.

The microscopic counts in the death phase are determined not only by the rates of death and autolysis, but also by the degree to which those cells still viable are multiplying; for the marked increase in the microscopic counts after the plate counts have reached their maximum can only be explained by the fact that some of the cells are still actively dividing and the progeny then soon dying. This occurred in greatest degree in the neutral medium, the maximum microscopic count showing an increase of 176 per cent over the maximum plate count. In the calcium chloride medium this

increase was 141 per cent, in the alkaline culture 58 per cent, in the acid 41 per cent, and in the salt agar 12 per cent. It is interesting in this connection that the first two cultures named also had a high incidence of constricted (dividing) cells.

At first an attempt was made, as in the preceding study, to follow variations in form by means of the area-length index. This, however, failed to yield any significant results, the figures so obtained showing no regular changes with increasing age of the culture, nor noteworthy differences when the averages for the various cultures were compared. It was quite apparent, however, by simply inspecting the slides that the important changes in cell form were not so much variations in relative slenderness or thickness as they were departures from the normal symmetry, i.e., development of budding, branching, bulging, and other unusual cell types. To measure the degree of variation in form then some scheme had to be adopted which would not only consider the relative slenderness and plumpness of the cells, but also these asymmetrical types, as well as curvature in the cells. What was desired was a quantitative expression of the degree of total variation encountered which could be compared directly with rates of death and autolysis determined as above.

This was accomplished in the following way. The cells were (more or less arbitrarily) divided into ten classes, illustrated in Figure 34. This illustration has been made by tracing from the cards, cells of the various classes from many different samples, simply to show the types of cells included in each class. They were chosen more or less at random, although an attempt was made to include extreme cases. It should be borne in mind that these composite fields do not indicate the frequency with which any of the cell types occurred in any particular culture; they are intended merely to indicate the nature of the classification followed. They are arranged and numbered in the order of their mean frequency in all of the cultures studied.

These ten classes include four classes of straight symmetrical cells: viz., slender rods (Class IV), normal rods Class I), oval cells (Class III), and spherical cells (Class VII). This is of course an entirely arbitrary division, since there occurs every degree of gradation from long filaments to perfectly round cells. But having

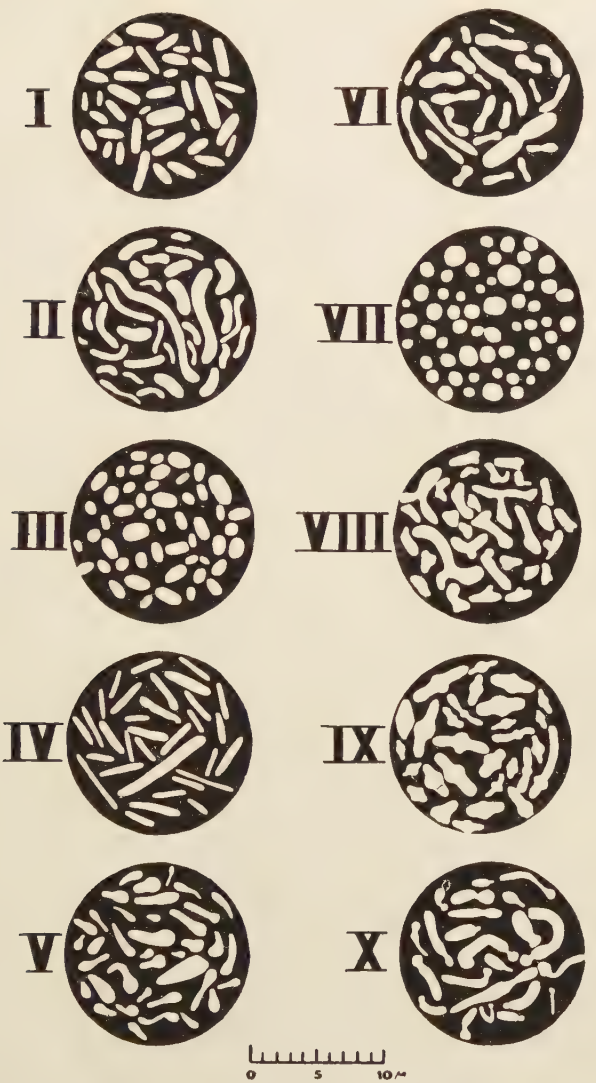

FIG. 34. REPRESENTATIVE CELLS ILLUSTRATING THE TEN CLASSES USED IN STUDYING INVOLUTION FORMS OF THE COLON BACILLUS.

From "The Involution Forms of Escherichia Coli." Through the courtesy of the Journal of Infectious Diseases. (1926, **39**, 429).

measured the area-length index, there was provided a means of separating these into classes which would not depend at all upon personal judgment. Area-length indices of 20, 40, and 60 were chosen as dividing points for these classes. Long, slender filamentous forms were considered as having an area-length index of 20 or less; normal forms from 21 to 40; oval forms from 41 to 60; spherical cells, those with an index higher than 60.

Further classes were provided as follows: All curved cells (Class II), regardless of their size or slenderness as long as there was a definite curvature to their axis; all cells which were greater in diameter at one end than the other (Class V), either provided with a knob-like expansion or showing a uniform graduation in diameter from the larger end to the smaller; all cells which showed a central constriction (Class VI), i.e., cells probably in the act of dividing; all cells which showed a lateral projection (Class VIII) whether in the form of a small bud or definite side branch; all cells which showed a sub-terminal constriction, i.e., which appeared to have a terminal bud (Class X); and finally all cells which exhibited a central bulging (Class IX).

Having decided upon this classification, all of the cards bearing the tracings of cells were gone through, and the proportion of the cells in each class determined for each sample. The average percentage of each class for all the the cultures at all stages of growth was then calculated, and the reciprocal of this percentage determined. The product of the average percentage incidence of any class by its reciprocal will of course equal one (or for convenience 100), and therefore the mean of these products for all ten classes will also equal 100. If now in any given sample the percentage frequency of each class is multiplied by the reciprocal of the percentage frequency of that class for the entire series of samples, and the average of these products for the ten classes is taken, there is obtained a value which indicates whether in that sample the degree of variation is greater or less than the average of all of the samples. Where there occurs more than the average number of the more frequent cell types (and therefore fewer of the less frequent types) this value will be less than 100, while conversely if there occurs more than the average number of the rarer cell forms the value will be greater than 100. This is really an

adaptation of the principle of index numbers to the problem at hand, the index being the mean variation in form of all of the cultures being compared. The value so computed may be called the index of variation of form. It is admittedly not an entirely satisfactory method, being rather artificial and involving to some extent personal judgment in classifying the cells, but in the absence of anything better may serve to supply at least an approximate quantitative expression of the degree of variation in form of the cells, which may be compared from day to day and from culture to culture.

The incidence of the various cell classes in the five cultures at various stages of growth is given in Table XXVII together with the indices of variation calculated from them. The mean frequencies of the ten cell types of the five cultures at all stages of growth are shown graphically in Figure 35.

The fluctuations of the index of variation day by day are somewhat irregular and do not appear to be highly significant; this is perhaps largely due to the small number of cells in each sample. The alkaline culture showed very little variation; there was a slight rise on the nineteenth day followed by a decline, corresponding with the period of decrease in death rate. In the neutral medium there occurred a well-defined increase reaching the maximum on the third day, corresponding with the beginning of the death phase; this was followed by a decrease and a second increase with its maximum on the fourteenth day, at which point the death phase had nearly reached its end, the number of viable cells remaining nearly the same during the remaining period of observation. The medium containing NaCl showed a very pronounced rise in the index of variation on the second day, due largely to the remarkably high incidence of budding and branching cells, followed by an irregular decline. The acid culture exhibited two maxima, one on the seventh day corresponding with the beginning of the death phase, and one on the nineteenth day when the death rate was decreasing. The medium containing $CaCl_2$ gave a slight initial rise followed by a decline, then a pronounced increase which was sustained until the nineteenth day, there being a marked reduction in the variation of the cells on the twenty-fifth day.

These fluctuations with time, while somewhat irregular and varying considerably in the different cultures, show in general a tendency

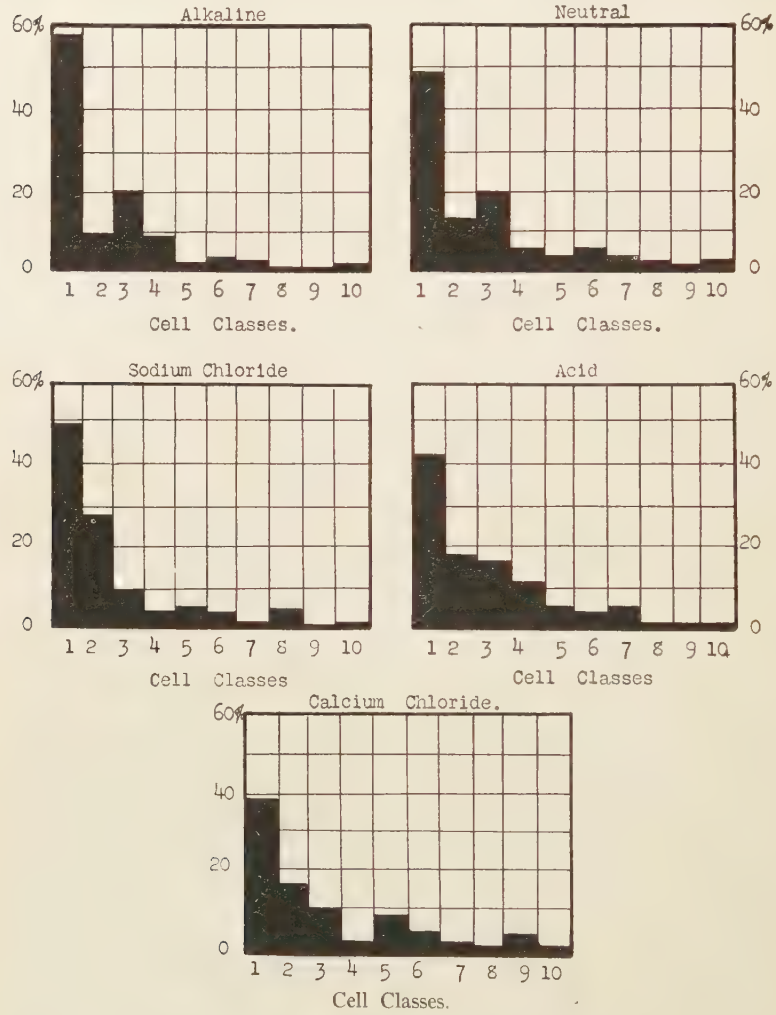

Fig. 35. Frequency Distributions of the Ten Cell Classes in Various Cultures of a Colon Bacillus.

to the formation of two maxima, one corresponding with the beginning of the death phase and one with the end. By computing the

mean index of variation of the five cultures for each day we increase the number of cases and obtain a correspondingly more accurate picture of the variations with time. There occurs a sharp rise at the beginning of the death phase followed by a decrease, then a second more sustained rise with another marked decrease on the last day. This final drop in the degree of variation in the form of the cells, occurring during the period of decreasing death rate as measured by the plate counts and of increasing rate as measured by the microscopic counts, can readly be explained by the final dissolution of those cells which presented the greatest departure from the "normal" form, and may be considered to support the argument that these irregular cells are actually dead. I am at a loss, however, to explain the initial rise and fall in the index of variation.

While the daily fluctuations in the index of variation did not prove very significant, and particularly there could not be established any clear relationship between the period of greatest degree of variation and the period of most rapid death, important results were obtained when the different cultures were compared with each other with regard to their mean indices of variation. In order of magnitude of this value they are arranged as follows: Alkaline (67.71), neutral (87.56), salt (93.01), acid (93.42), and calcium chloride (157.25). It will be noted that the values for the salt and acid cultures are nearly identical. But the figures for the salt culture are not directly comparable with the others, as no observation was made on the twenty-fifth day. Since in all of the cultures there was observed a decrease in variation on the last day, it is probable that this would also have occurred in the medium with sodium chloride, and that the mean index of variation would have been somewhat reduced.

The rank order of the cultures with regard to their mean indices of variation does not correspond with their rank order as regards mean rate of death, maximum rate of death, or favorableness of the medium for growth. In fact the cultures containing calcium chloride, which gave the highest degree of variation in cell form, had nearly the lowest death rate. It is clear therefore that there cannot be established a correlation between the index of variation and the rate of death.

This rank order does, however, correspond definitely with the

rank order of the cultures according to their rates of autolysis, the alkaline culture which required the longest time for half of the dead cells to become stainable being the one with the lowest index of

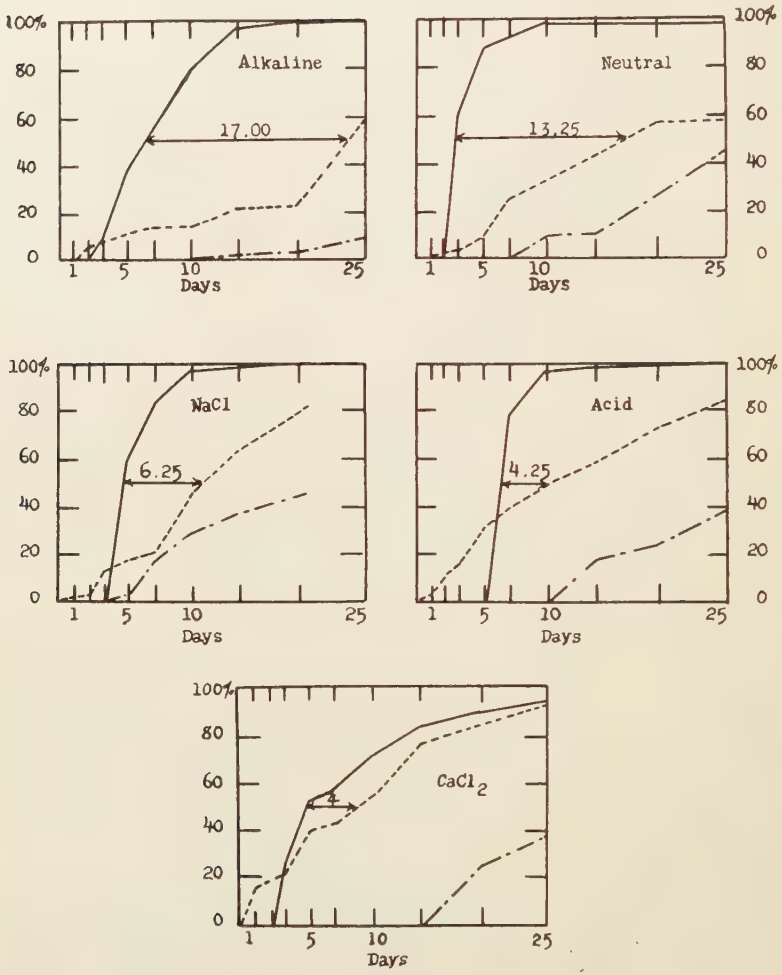

FIG. 36. RATES OF DEATH AND AUTOLYSIS IN VARIOUS CULTURES OF A COLON BACILLUS.

Solid lines indicate percentage decrease in plate counts; dotted lines, percentage increase in stained cells; dot-and-dash lines, percentage decrease in microscopic counts.

variation, and the calcium chloride culture with the highest autolysis rate having also the highest index of variation. This is of course only a rank order correlation, i.e., the figures for autolysis rate and degree of variation in cell form vary in the same direction, but not proportionately. But considering the imperfect nature of the measures both of rate of autolysis and variation in form, I believe that this correlation is significant, and offers a real clue as to the nature of the unusual cell forms observed during the death phase.

It was pointed out in Chapter V that probably the form of bacterial cells is maintained by a certain degree of rigidity of the cell walls. If this membrane be softened or destroyed locally, it will lead to a local bulging of the protoplasm, forming lateral or terminal buds or branches or central bulgings depending upon the localization of the area of destroyed or altered cell wall; while if the process involves the entire cell it will give rise to oval and eventually spherical forms. If autolysis be delayed, the dead cells will tend to maintain their form even though the death rate be high. But if autolysis is rapid, and alters the cell wall in the manner suggested, variation in form will be great even though the death rate is relatively low.

While the differentiation between stained and unstained cells is quite easily made by inspection through the microscope, there is not sufficient contrast in the photographic negative when projected at the magnification used to certainly separate the two classes when making the tracings. Consequently no comparison can be made of the variation in form between the stained and unstained cells. Microscopic observation indicates, however, that the majority of the unusual forms, as budding, branching, and bulging cells, are not stained. This, however, does not necessarily indicate that the variation in form is not due to the same process which leads to the cells becoming stainable; the changes in the cell membrane may occur in an earlier stage of the process.

CHAPTER X

ON CYTOMORPHOSIS IN BACTERIA

The collection of these data has proven exceedingly tedious and time-consuming. The observations recorded in the preceding pages have required measurements of one character or another of nearly 100,000 cells. For this reason the data are quite incomplete. Much more work will have to be done, many more species of bacteria will have to be studied on a greater variety of media, before the nature and significance of their morphologic variations can be clearly understood in all details. But enough has been presented, I believe, to indicate clearly that the problem includes factors not recognized by either monomorphists or pleomorphists. The cells of bacteria *do* vary in size and form and internal structure, but these variations occur in a very regular and orderly fashion; they can be measured, and the quantities obtained by such measurement when plotted form regular curves. These variations are not confined to the late stages of growth but occur continually through all stages. Each character reaches its maximum development in some particular phase or at some particular point of inflection of the growth curve. Those factors which cause a variation in the growth rate equally vary the rate and degree of change in morphologic characters. The morphologic variations of bacteria are therefore an expression of the variations in growth rate.

The character of these morphologic variations in the early stages of growth depends largely upon the age of the cells used for seeding. If these were embroyonic forms rapidly growing, they continue as such in the new medium. If, however, they were of a later stage of development, they may remain dormant for a time and even continue to change in the same direction as in the old culture from which they came. Thus in Cultures II and III of *B. megatherium* described in Chapter IV, the cells decreased slightly in size during the first hour of growth; with the diphtheriod organism recorded in Chapter VI there was an initial increase in size; with the cholera

vibrio as described in Chapter VIII there was at the beginning a slight decrease in size and increase in area-length index. While these were not in all cases associated with dormancy of the cells as regards division rate, it seems to me that we are dealing with the same sort of phenomenon; the cells do not immediately recover from the aging influence which was present in the old culture.

It would seem from my data that the division of the growth curve into a lag phase (of accelerating growth), a logarithmic growth phase, and a resting phase, is not so significant as a division into a phase of accelerating growth and a phase of negative acceleration in growth; the so-called logarithmic growth phase when present is but a long drawn out point of inflection. For the morphologic variations which occur during the early stages of growth progress definitely to this "mid-point" (as Pearl designates it) of the growth cycle, then turn sharply in the opposite direction. The embryonic forms reach their maximum development just at the beginning of negative acceleration in growth rate, the mature forms at the end of growth. The transformation of aged cells to embryonic forms precedes cell division; the initial period of dormancy then is occupied in part (but only in part) by this transformation. The gradual acceleration in growth rate is probably best explained by the nature of the frequency distribution of the inoculated cells with regard to their capacity for growth, as reflected in their frequency distributions with regard to those morphologic characters which distinguish the embryonic forms.

These embryonic forms will vary in their characters with different species of bacteria, but it is apparent from what has been presented here, as well as from the observations of Clark and Ruehl, that with most forms, especially the rods, they differ from the mature forms particularly in increased length and slenderness. The diphtheroid group are apparently an exception, the embryonic forms being shorter and more nearly approaching the spherical form. In all cases these embryonic forms seem to possess a higher affinity for the basic aniline dyes. In those cases where the young cells show an increased size there is also apparent an increased variability in size. In those forms which develop intracellular granules or other structures these are lacking in the embryonic cells. In

the case of the cholera vibrio, which may perhaps be taken as a type of the spiral organisms, the embryonic forms are characterized particularly by straightness of their cells; they are bacillary in form.

The mature or differentiated forms, beginning to develop with negative acceleration in growth and reaching their maximum at the end of growth, are just the reverse of the embryonic forms in the characters enumerated above; in most species the cells are shorter and more nearly approach the oval or spherical form; they are less variable in size, more variable in form. In the diphtheriod group they are longer and more slender. With the cholera vibrio they are particularly characterized by curvature of the cells. In all forms they are less deeply stained by the basic dyes. It is of course obvious that these cells are not differentiated in the sense that different cells show a great diversity of form and internal structure as occurs in the differentiated cells of a multicellular organism; such cannot be the case because the cells are all contained in the same environment which must be nearly uniform throughout. But the individual cells do show a differentiation in their internal structure, many forms developing within the protoplasm spores or granules of one type or another, especially volutin. Now it is just this development of internal "paraplasmatic" structures which characterize the differentiated cells of a multicellular organism, and which are either the result or the cause of their diversified function. In this sense at least, then, there does occur differentiation in the mature cells of bacteria.

The senescent forms begin to develop with the initiation of the death phase. Perhaps because the data are too incomplete their relationship to this phase of growth is not so clear as in the case of the other two cell types; but it would seem that they are not so definitely associated with the rate of death as with the rate of autolysis during the death phase. They are characterized by increased variation in form of cells and especially by an increasing proportion of asymmetrical cells. I have already suggested that this is due to the action of some agent upon the cell wall. These senescent forms are also characterized by still less affinity for the basic dyes and finally by the development of an affinity for the acid dyes.

Throughout the preceding pages I have tried wherever possible to call attention to the parallelism in the phenomena of growth and the accompanying cell changes existing between cultures of bacteria and multicellular organisms. While many biologists have studied the cell changes of plants and animals which occur during growth, senescence, and death, their significance has been especially emphasized by Minot, who named the process "Cytomorphosis."

In animals this cytomorphosis is characterized, according to Minot, especially by variations in the ratio of size of the nucleus to volume of protoplasm. This ratio is high in the embryonic cells during the period of most active growth, and becomes progressively lower with increasing age of the animal, the cells becoming increasingly larger without any corresponding increase in size of the nucleus, although, as he points out, these differentiated cells are also characterized by the occurrence of various structures within the protoplasm; the increase in size of the cell being to a certain extent at least due to the accumulation of these granules, vacuoles, and similar structures. Senescence is but a continuation of the same process, the nucleus continuing to shrink. The protoplasm may either atrophy or hypertrophy, according to the nature of the cell, and frequently also still further types of granules or other structures may appear.

According to Minot, this process is due to intrinsic causes. Once a cell has been started on the growth cycle, it must necessarily pass through the remaining phases of this cycle. Growth, senescence, and death are but outward manifestations of this cytomorphosis. As he states it, "the increase of protoplasm together with its differentiation is to be regarded as the explanation (or should we say cause) of senescence." Further, the process is to a degree irreversible; "after a cell has progressed and is differentiated a certain distance, its fate is by so much determined." This he calls the "law of genetic restriction."

In the light of later knowledge, however, these last statements can hardly be accepted. The present day tendency is rather to consider the changes which occur in the cells of a growing plant or animal not so much a cause of growth as a result. They are morphologic manifestations of physiological changes which are occurring

in the organism. As an extreme example of this viewpoint, we may quote Child, who maintains that what we call protoplasm of cells is but a relatively inert framework about which the chemical processes concerned with growth and age and death take place. He states that, "According to this view the colloid substratum and the morphologic structure of the organism represent, so to speak, the sediment of the metabolic process."

Also the widespread occurrence of dedifferentiation in cells, not generally admitted at the time of Minot's publications, now seems to be generally accepted. This is especially evident in lower forms of life, in the process of regeneration after excision of a part or in the reconstitution of individuals from excised parts. It also seems to have been established beyond question in the case of tissue cultures. Here conditions are quite comparable with those which obtain in cultures of bacteria. Cells left remaining in plasma tend to differentiate and finally undergo senescence and death; while if continually transferred to fresh plasma they may be continually maintained in a relatively undifferentiated condition. Similarly, as was shown in Chapter IV, bacteria left in the same medium tend to revert to the mature form as indicated by shorter length of cells, but if continually transferred to fresh agar remain in the embryonic form characterized by long cells.

Child has studied the phenomena of growth and death from a physiologic standpoint, and has discovered that the physiologic age of an organism or tissue can be determined by its relative susceptibility to certain poisons, there being a correlation between the degree of susceptibility and the degree of physiologic youth of the cells or organisms investigated. Not only is there a difference in the susceptibility between young and old individuals, but it can be shown that there is a difference in susceptibility between growing and resting parts of an organism. Regenerating pieces of planarian worms are physiologically younger than the same tissues when a part of the whole animal; and similarly small pieces as measured by their susceptibility have a higher metabolic rate than large ones. This is very interesting from our standpoint, because, as has been previously related, Sherman and Albus have discovered that cultures of bacteria are more susceptible to various injurious agents, as heat

and cold and salt and phenol, than are resting cultures; that is, there is with bacteria a physiologic youth that can be demonstrated by the same means as physiologic youth in a growing animal or plant.

I have also shown previously that cultures which are lightly seeded develop a higher growth rate and a greater degree of morphologic variation in the embryonic cells than cultures which are heavily seeded, and have compared this with the higher growth rate in regenerating tadpoles' tails when only a small stump is left. It would appear, therefore, that whether measured by degree of morphologic variation in the cells, by the actual rate of cell division, or by physiological processes, such as the susceptibility to poisons, the growth rate both of free populations of cells, and of multicellular organisms is dependent upon the relative volume of protoplasm which is in the process of growing.

While there have been made some quantitative studies of the nucleo-cytoplasmic ratio of cells at different stages of growth in developing animals, I am not aware of any quantitative investigations of cytomorphosis in multicellular organisms comparable with those which I have presented for bacteria, by which the degree of change in the cells can be directly correlated with the rate of growth. There is, however, ample indirect evidence that such a correlation exists. Minot noted that the "mitotic index," i.e., the percentage of cells in mitosis (serving as a measure of rate of cell division), steadily decreases with increasing differentiation, and that the rate of differentiation becomes increasingly slower with age as does also the growth rate. Child observed that the degree of differentiation or dedifferentiation of the cells was correlated with the metabolic rate as determined by susceptibility, which in turn was correlated with the rate of growth. It would seem, therefore, not improbable that the transition from embryonic to differentiated cells in multicellular organisms is determined by a change from positive to negative acceleration in growth rate. A quantitative determination of such a correlation, however, would present considerable technical difficulties.

Minot predicted that with further study, cell cycles in one celled organisms, protozoa, would be found to be of the same nature as

the cytomorphosis which occurs in multicellular organisms; that with increasing age in protozoa a change in the nucleo-protoplasmic ratio would be found comparable with that which occurs in differentiating animal and plant cells. While I am not conversant with the literature, I do not know that this has been done, that is that the life cycles in protozoa have actually been studied as a cytomorphosis, and that any attempt has been made to trace in them a development of the same laws which govern the cell changes in the multicellular organism; but I believe that in the data which have been presented in the preceding chapters of this book there is ample evidence that the cell changes exhibited by bacteria are of this nature, and that this term "cytomorphosis," with the present state of our knowledge at least, more clearly explains the nature of the variations in cell structure which are observed, than does the term "life cycle," since it does not necessarily involve any idea of multiplicity in forms of reproduction, and particularly of alternating sexual and non-sexual phases which generally is implied in the term "life cycle."

My conclusion that the cell changes occurring in cultures of bacteria are a cytomorphosis of the same kind as that exhibited by the cells of a multicellular organism is arrived at only by analogy. No one can state definitely that the growth and cytomorphosis of a population is governed by laws identical with those which govern the growth and cytomorphosis of a multicellular plant or animal until it has been discovered what those laws are. But the phenomena so exactly parallel each other, no matter from what angle they are viewed, the analogy is so perfect, that we are justified in accepting this theory as at least a working hypothesis for further investigation.

The acceptance of this theory demands the acceptance of certain corollaries. If it be granted that the cells of bacteria undergo a metamorphosis of the same kind as that exhibited by multicellular organisms, then it must be granted that to this degree a population of free one-celled organisms, even though those cells have no connection other than the common nutrient fluid which bathes them, behaves like an individual. There has already been accumulated a great deal of evidence of other kinds to support the idea that there is no essential difference, that there can be drawn no hard and

fast line, between populations of one-celled organisms and multicellular individuals; that a higher plant or animal is but a population of more highly differentiated cells. But there has been, in the past at least, a tendency to look upon cell differentiation in multicellular organisms as being the result of some organizing agency peculiar to such individuals. If, however, we find in cultures of microörganisms where no such governing agency can be supposed to exist, a differentiation of cells, even though very primitive, we are forced to conclude that such is not the case; that the high degree of organization of higher organisms is a result and not a cause of the high degree of cell differentiation.

This theory of cytomorphosis in bacteria is not entirely in disagreement with the current theory of monomorphism, which has always recognized two of the three cell types; the so-called normal forms which I designate mature or differentiated, and the involution forms which I call senescent. It differs markedly, however, in the recognition of a third type, the embryonic cells characteristic of the stage of accelerating growth which will probably be found in all species of microörganisms; but just this recognition of the embryonic forms is sufficient to upset the entire theory of monomorphism, for it implies that morphologic variations are occurring continuously at all stages of growth, rather than only during the period when cultures are becoming aged and dying. It would seem, therefore, that the monomorphistic theory without modification can no longer be considered sound.

On the other hand, this theory of cytomorphosis is not necessarily opposed to the theory of complex life cycles maintained by the more recent pleomorphists. The two viewpoints are rather different ways of looking at the same thing than are they mutually exclusive. But with acceptance of the theory of cytomorphosis we are no longer forced to explain every variation encountered as some sort of a mode of reproduction other than simple cell division. Most of the cell types described as extraordinary reproductive bodies by the pleomorphists, I have encountered only in the death phase, and their occurrence is apparently dependent on the rate of autolysis; it would seem, therefore, more likely that they are cells which are undergoing retrogressive changes than cells which are embarking upon some

new mode of growth. But we have every evidence that in other organisms modes of reproduction other than simple vegetative division or budding are also dependent upon the age of the culture. As Child states, "there is good reason to believe that algae and fungi may undergo senescence and rejuvenescence like the lower animals, and that the different forms of reproduction are characteristic of different stages of the life cycle" (i.e., phases of growth). It is quite possible then that bacteria do develop during the death phase some sorts of reproductive bodies that give rise to particular forms of resting cells; that there does occur, as Mellon claims, some sort of a reorganization process. But since we know that in higher organisms along with these reproductive processes there occur retrogressive changes in the vegetative cells, we are justified in demanding conclusive proof that these unusual cells found in the death phase are really reproductive cells and not simply degenerating cells. The burden of proof of complex life cycles in bacteria must be placed upon the shoulders of those men who have declared that such life cycles exist. So far such proof has not been brought forward.

In connection with these theories of pleomorphism, it should also be pointed out that every study of life cycles of bacteria should be accompanied by observations of the rate of growth and a determination of the phase of growth in which each type of cell change occurs. The age of the culture is of paramount importance in interpreting the nature of the cell variations which are observed, and by this I mean physiologic age rather than age in hours. It is not sufficient to state that a culture is so many hours or days old, for the rate of growth may be varied by many factors. As has been shown in the earlier part of this work, during the growth phase cells may change in size within one or two hours to such a degree that they would by ordinary standards not be recognized as the same. One cannot, therefore, safely guess at the phase of growth; it must be determined by measurement. Many of the morphologic variations which have hitherto been described, particularly those indicating a possible mutation in the organisms studied, have undoubtedly been not true permanent variations but the result of a comparison by the investigator of cultures which are in

different phases of growth. The diphtheriod organism which I have studied, for instance, looks almost like a streptococcus in the period of active growth, while it is a long, granular rod in the resting phase. Now if this organism had been inoculated into different media, in one of which it grew slowly and the other rapidly, and these were compared, it would seem to the observer that the organism had undergone a pronounced variation, and this would be apparent every time the organism was subcultured on to these two media, other conditions being the same. It is, therefore, necessary to determine the phase of growth in every case where cultures are compared with the view to establishing mutation.

It should also be pointed out here that those who may attempt to repeat my experiments must inoculate their cultures lightly, if they will see the degree of morphologic variation in the early stages of growth which I have described. By the ordinary procedure of transferring from one agar slant to another, generally a loopful of the organism is carried over. A culture so inoculated will have an extremely short period of accelerating growth, during which only slight change in cell size will be observed. The difference between the embryonic forms and mature forms is quantitative, and this difference becomes greater the higher is the actual rate of growth attained.

This chapter should not be closed without a word or two regarding the relationship of these findings to problems of general bacteriology. The morphologic variations described are but outward manifestations of equally profound variations in the physiology of the organisms, concerning which we are practically in the dark. There are scattered references in the literature to differences in degree of virulence, of antigenic value, of susceptibility to antibody action, or differences in fermentative power, between young and old cultures; but in practically no cases have these variations in physiologic properties been definitely correlated with phases of growth. It would seem that this would offer an extremely attractive field for investigation, especially from the standpoint of infection and immunity. Such investigations, however, are difficult to carry out, because when the cells of bacteria are physiologically young, they must necessarily be few in number in proportion to the volume of

the medium, so few that they are not suitable for most types of experiment. Every attempt to concentrate them, particularly to remove them from their medium, immediately causes them to rapidly age, and they are no longer physiologically young cells but mature cells. For this reason I do not believe that very much experimental work has so far been done with truly physiologically young bacteria. Some way must be discovered for either working with small numbers of cells in the medium in which they are growing, or else concentrating them in some way that their properties are conserved, before much can be accomplished in this direction.

APPENDIX

TABLES I TO XXVII

APPENDIX

Table I

Influence of Size of Seeding on Growth Curve of Yeast

Hours of incubation	Number of Yeast Cells per c.c. of Medium (10% dextrose, 2% peptone)				
	Flask No.1	Flask No. 2	Flask No. 3	Flask No. 4	Flask No. 5
0	20,949,750	2,094,975	209,498	20,950	2,095
2	23,809,916	2,223,833	221,500	17,200	2,660
4	37,816,500	3,109,500	212,400	25,300	3,100
6	58,944,000	8,333,000	357,500	20,700	3,500
8	105,568,000	19,683,000	1,033,800	20,300	4,000
10	129,008,000	31,675,000	3,794,000	24,700	4,000
12	161,600,000	54,550,000	11,839,000	41,100	4,800
16	174,720,000	91,544,000	44,848,000	259,000	4,800
20	176,264,000	118,040,000	89,368,000	3,004,200	5,500
24	181,008,000	124,880,000	123,768,000	18,355,200	6,600
36	183,680,000	144,128,000	138,304,000	114,600,000	289,400
48	186,088,000	156,752,000	146,704,000	134,984,000	1,089,600
72	185,160,000	165,648,000	162,240,000	160,000,000	93,264,000
96	195,008,000	173,536,000	176,320,000	165,176,000	169,400,000
144	197,896,000	183,248,000	180,368,000	175,840,000	171,408,000

Table II

Influence of Concentration of Nutrients on Growth Curve of Yeast

Hours of incubation	Number of Yeast Cells per c.c. of Medium				
	Flask No. 1	Flask No. 2	Flask No. 3	Flask No. 4	Flask No. 5
	10% Dextrose, 2% Peptone	5% Dextrose, 0.4% Peptone	2.5% Dextrose, 0.08% Peptone	1.2% Dextrose, 0.016% Peptone	0.6% Dextrose, 0.0032% Peptone
0	20,800	21,000	20,500	20,800	18,800
2	22,100	21,400	22,700	22,600	22,300
4	24,200	22,800	26,400	28,400	17,400
6	28,200	77,600	57,200	29,000	29,000
8	50,000	190,300	134,900	61,000	31,200
10	82,300	404,700	224,400	102,000	33,300
12	310,800	1,029,200	375,200	131,800	43,000
16	3,749,600	4,502,200	422,000	191,100	50,200
20	28,241,600	12,016,000	682,400	271,200	56,400
24	62,480,000	18,248,000	1,375,500	272,900	62,200
36	173,280,000	28,825,600	3,402,200	389,700	65,100
48	177,440,000	33,760,000	4,012,200	496,300	92,000
72	215,728,000	35,728,000	5,544,400	500,500	89,500
96	237,400,000	47,050,000	6,472,000	543,600	100,300

TABLE III

MICRO-COLONY I

RATE OF GROWTH

Minutes of growth	No. of cells in colony	Average length of cells-microns	Sum of lengths of cells-microns
0	2	3.7	7.5
30	2	4.7	9.4
45	2	4.7	9.4
100	2	5.6	11.2
120	2	6.6	13.1
150	2	8.8	17.5
165	2	9.2	18.5
180	2	10.6	21.3
195	2	13.1	26.3
210	2	12.8	25.6
225	3	12.1	36.3
240	4	9.8	39.4
255	4	11.6	46.3
270	4	17.3	69.4
285	4	16.9	67.5
300	7	11.0	76.9
315	9	12.1	109.4

TABLE IV

MICRO-COLONY II

RATE OF GROWTH

Minutes of growth	No. of cells in colony	Average length of cells-microns	Sum of lengths of cells-microns
0	2	6.6	13.1
120	3	5.6	16.9
180	4	5.5	21.9
210	4	5.1	20.6
225	5	5.2	26.2
240	5	6.1	30.6
270	5	7.5	37.5
280	5	10.0	50.0
295	6	8.9	53.7
310	7	8.9	62.5
325	8	8.0	63.7

TABLE V

MICRO-COLONY III

RATE OF GROWTH

Minutes of growth	No. of cells in colony	Average length of cells-microns	Sum of lengths of cells-microns
0	5	4.6	23.1
30	5	4.9	24.4
75	5	4.3	21.3
105	5	5.1	25.6
120	6	4.9	29.4
135	7	4.9	34.4
150	8	5.4	43.1
165	8	5.8	46.3
180	8	6.9	55.0
195	11	5.8	63.8
210	13	6.2	80.6
220	16	6.7	108.1

TABLE VI

B. MEGATHERIUM, CULTURE I

RATE OF GROWTH

Hours of growth	No. of cells per cu. mm.	No. of spores per cu. mm.
0		
½	90	
1	110	
1½	139	
2	169	
2½	178	
3	196	
3½	209	
4	244	
4½	416	
5	1,370	
5½	2,080	
6	5,920	
6½	10,380	
7	15,040	

TABLE VI (*Continued*)

Hours of growth	No. of cells per cu. mm.	No. of spores per cu. mm.
7½	18,540	
8	21,080	
8½	38,409	
9	57,240	
9½	79,700	
10	63,200	
10½	77,900	
11	76,800	
11½	125,000	
12	136,450	
13	143,400	
14	171,600	
15	183,500	
16	220,500	
17	225,500	20
18	242,500	120
19	244,500	420
20	264,400	1,520
21	318,000	8,400
22	258,000	10,800
23	192,000	27,600
24	163,200	38,400
28	156,000	102,000
32	141,600	140,400
36	138,000	181,200
40	121,200	201,000
44	96,000	216,000
48	93,000	234,000
60	78,000	315,000
72	58,000	316,000

TABLE VII

B. MEGATHERIUM, CULTURE I

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	3.4	3.0	3.1	0.80	23
2½	3.9	3.5	3.5	1.38	35
3	5.3	4.0	4.7	2.20	42
3½	6.5	3.5	5.5	3.58	55
4	9.1	3.5–4.0	10.2	5.55	61
4½	7.3	3.5	5.4	4.70	64
5	11.3	11.0–12.0	10.6	6.16	54
5½	19.8	14.5–15.0	16.8	8.67	43
6	13.0	10.5	12.8	4.42	34
6½	15.0	14.0	14.2	5.92	37
7	9.0	7.5	8.4	3.12	34
7½	7.2	5.5	6.5	2.89	32
8	5.7	5.0	5.3	1.40	24
8½	5.0	4.0–4.5	4.4	1.87	36
9	4.3	3.5	3.7	1.98	46
9½	4.7	3.5	3.7	1.49	32
10	4.5	4.0	4.0	1.02	23
10½	4.0	4.0	3.7	0.90	22
11	3.6	3.5	3.3	0.90	25
11½	3.4	3.5	3.1	0.69	20
12	3.4	3.0	3.1	0.80	23
13	3.9	4.0	3.6	0.86	22
14	4.1	3.5–4.0	3.7	0.90	21
15	3.8	4.0	3.6	0.86	22
16	4.3	4.0	4.0	1.06	24
17	4.1	4.0	3.7	0.93	23
18	4.1	4.0	3.8	0.85	21
19	4.0	4.0	3.7	0.80	20
20	3.8	3.5	3.5	0.95	25
21	4.5	4.0	4.2	1.10	24
22	4.1	4.0	3.8	0.99	24
23	4.6	4.0	4.2	1.15	25
24	4.2	4.0	3.9	0.92	22
28	4.0	4.0	3.8	0.81	20
32	3.6	3.5	3.2	0.86	24
36	3.8	4.0	3.5	0.83	22

TABLE VII (*Continued*)

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
40	3.4	3.5	3.1	0.76	22
44	3.2	3.0	2.9	0.79	24
48	3.6	3.5	3.3	0.80	22
60	3.9	3.5	3.6	1.15	29
72	2.8	2.5	2.4	0.82	29

TABLE VIII

B. MEGATHERIUM

RATE OF GROWTH

Culture II		Culture III	
Hours of growth	No. of cells per cu. mm.	Hours of growth	No. of cells per cu. mm.
1	2,340	1	240
2	3,120	3	2,520
3	6,910	4	3,670
4	8,340	5	4,110
6	10,620	6	5,100
8	11,800	7	5,740
10	12,280	9	6,240
12	15,900	10	6,920
14	18,630	13	8,150
16	21,120	15	10,400
18	30,810	16	13,740
20	43,830	18	16,110
22	52,830	20	28,230
24	54,760	22	36,200
		24	43,820

TABLE IX

B. MEGATHERIUM, CULTURE II

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	5.2	5.0	4.8	1.4	27
1	4.9	4.5	4.5	1.2	25
2	6.6	5.5	6.1	1.9	29
3	7.6	6.5	7.1	2.1	27
4	8.5	7.5	7.9	2.7	31
5	7.6	7.0	7.1	2.3	31
6	7.0	6.5	6.4	2.3	31
7	6.8	5.5	6.1	2.2	32
8	6.5	5.5	5.9	2.1	32
9	5.9	5.0–5.5	5.4	1.7	29
10	5.5	5.0	5.2	1.6	31
11	5.1	5.0	4.7	1.3	25
12	5.1	4.5	4.6	1.6	31
13	5.0	4.0	4.4	1.7	34
14	4.6	4.0	4.2	1.4	31
16	4.5	4.0	4.2	1.4	31
18	4.1	3.5	3.6	1.5	37
20	3.6	3.5	3.3	1.1	30
22	3.5	3.0	3.1	1.2	33
24	3.6	3.0	3.1	1.2	34

TABLE X

B. MEGATHERIUM, CULTURE III

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	5.2	5.0	4.8	1.4	27
1	5.0	4.5	4.6	1.2	24
2	6.9	6.0	6.3	2.0	30
3	9.3	7.5	8.8	2.7	28
4	9.7	8.5	9.0	2.8	29

TABLE X (*Continued*)

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
5	9.6	7.5–8.0	8.9	2.9	31
6	9.3	9.0	8.8	2.8	30
7	8.7	8.5	8.2	2.8	33
8	6.9	6.0	6.4	2.0	28
9	7.3	6.0	6.8	2.2	30
10	5.9	5.0	5.3	2.1	36
11	6.2	5.5–6.0	5.8	2.0	32
12	5.1	4.5	4.5	1.8	35
13	4.9	4.0	4.4	1.5	30
14	4.6	4.0	4.2	1.4	30
16	4.7	4.0	4.2	1.5	32
18	4.4	3.5	3.9	1.6	35
20	4.4	3.5	3.9	1.5	33
22	3.6	3.5	3.3	1.1	31
24	3.8	3.5	3.4	1.2	33

TABLE XI
B. MEGATHERIUM
RATE OF GROWTH

Culture IV		Culture V		Culture VI	
Hours of growth	No of cells per cu. mm.	Hours of growth	No. of cells per cu. mm.	Hours of growth	No. of cells per cu. mm.
0	520	0	48	0	(5)*
1	662	1	59	3	33
2	990	3	225	5	340
4	1,980	4	405	6	1,040
5	2,450	5	1,620	7	1,560
6	3,170	7	2,290	9	2,250
8	4,030	8	2,610	10	2,680
10	4,480	9	3,120	12	2,920
12	4,800	12	4,180		

* It was impossible to make accurate counts in this series with samples earlier than three hours. Since this series was inoculated with 1/100th as much material

as Culture IV, which contained 520 cells per cu. mm. at 0 hour, it is assumed that 5 cells per cu. mm. is a fair estimate of the number at the beginning of growth in Culture VI.

TABLE XII

B. MEGATHERIUM, CULTURE IV

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	5.8	5.0	5.3	1.7	28
1	5.6	5.0	5.0	1.8	32
2	5.3	4.5	4.7	1.9	36
4	6.1	5.0	5.6	1.8	30
5	6.0	4.0	5.1	2.5	42
6	7.3	6.0	6.8	2.2	31
8	7.5	7.0	7.0	2.2	30
10	7.0	5.5	6.2	2.3	33
12	7.3	6.0	6.6	2.6	35

TABLE XIII

B. MEGATHERIUM, CULTURE V

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	5.8	5.0	5.3	1.7	28
1	4.3	4.0	3.9	1.2	28
3	5.3	4.5	4.7	1.8	34
4	6.3	5.5	5.8	1.7	28
5	8.0	7.0	7.5	2.1	26
7	7.2	6.5	6.6	2.0	28
8	7.1	6.0–6.5	6.6	2.0	27
9	8.3	8.5–9.0	8.0	2.0	24
12	9.0	7.0	8.5	2.6	28

TABLE XIV

B. MEGATHERIUM, CULTURE VI

STATISTICAL CONSTANTS

Hours of growth	Average (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	5.8	5.0	5.3	1.7	28
2	4.5	4.0	4.1	1.0	23
3	5.1	4.5–5.0	4.8	1.2	23
5	7.3	6.0	6.8	2.2	30
6	7.5	8.0	7.1	2.2	29
7	8.6	5.0	7.8	3.4	39
9	8.5	8.5–9.0	8.1	2.3	26
10	7.0	6.0	6.3	2.2	31
12	6.9	6.0	6.3	1.8	26

TABLE XV

EFFECT OF CONCENTRATION OF NUTRIENTS ON SIZE OF CELLS (B. MEGATHERIUM)

Hours of Growth	Average (microns)	Standard Deviation (microns)	Coefficient of variation
Full Strength Agar			
0	4.4	1.4	32
1	5.2	1.9	37
2	7.2	1.9	27
3	8.9	2.3	26
4	8.8	2.7	31
5	4.6	1.2	27
6	3.3	.8	23
7	3.2	.9	30
8	3.2	.8	25
Half Strength Agar			
0	4.4	1.4	32
1	4.9	1.6	32
2	7.2	1.8	25
3	8.6	1.9	23
4	4.2	1.1	27
5	4.5	1.8	40
6	3.4	1.2	35
7	3.3	.7	21
8	2.8	.7	25

TABLE XV (*Continued*)

Hours of Growth	Average (microns)	Standard Deviation (microns)	Coefficient of variation
Quarter Strength Agar			
0	4.4	1.4	32
1	4.9	1.6	30
2	6.5	1.8	28
3	6.2	2.3	37
4	4.1	1.2	30
5	3.7	.9	27
6	3.1	.7	22
7	2.7	.6	24
8	2.5	.7	28
Eighth Strength Agar			
0	4.4	1.4	32
1	4.9	2.1	46
2	7.2	2.2	30
3	6.5	2.0	30
4	3.9	1.0	27
5	2.9	.7	22
6	2.7	.6	22
7	2.6	.6	25
8	2.4	.6	27
Sixteenth Strength Agar			
0	4.4	1.4	32
1	4.8	1.5	30
2	6.1	1.7	28
3	6.0	1.9	32
4	4.3	1.4	32
5	3.3	1.1	32
6	3.6	1.3	38
7	3.3	1.0	32
8	2.9	.8	29

TABLE XVI

EFFECT OF AGE OF PARENT CULTURE ON SIZE OF CELLS (B. MEGATHERIUM)

Hours of Growth	Average (microns)	Standard Deviation (microns)	Coefficient of variation
	Parent Culture		
0	3.4	1.2	36
1	4.5	1.7	39
2	4.9	1.8	37
3	7.6	2.7	36
4	8.0	2.3	29
5	6.6	1.7	26
6	3.7	.9	25
7	3.6	1.0	28
8	3.1	.9	28
	Two-hour Subculture		
0	4.9	1.8	37
3	8.4	2.6	31
4	7.4	1.9	26
5	6.9	2.2	32
6	4.0	1.2	31
8	3.7	1.4	37
	Four-hour Subculture		
0	8.0	2.3	29
1	8.6	2.6	30
2	9.5	3.0	32
3	9.5	2.7	28
4	8.2	2.4	29
5	7.1	3.0	42
6	4.1	1.3	33
7	3.3	.7	22
8	3.3	.8	25

TABLE XVI (continued)

Hours of Growth	Average (microns)	Standard Deviation (microns)	Coefficient of variation
Six-hour Subculture			
0	3.7	.9	25
1	4.4	1.5	33
2	5.7	1.8	31
3	7.9	1.9	24
4	9.5	2.1	22
5	7.2	2.0	27
6	4.9	1.7	34
7	4.0	1.3	33
8	3.2	.7	23
Eight-hour Subculture			
0	3.1	.9	28
1	3.5	1.0	29
2	4.1	1.1	27
3	6.0	1.8	30
4	8.3	2.3	27
5	7.7	2.0	26
6	4.2	1.2	28
7	3.5	1.1	31

TABLE XVII

B. COLI, RATE OF GROWTH

Hours	Cells per cu. mm.
0	40,400
3	371,600
6	1,592,000
9	2,466,800
12	3,060,400
18	3,190,000
30	3,577,200
72	3,792,400
96	4,594,400

TABLE XVIII

B. COLI, STATISTICAL CONSTANTS

Hours	Average length (microns)	Standard deviation (microns)	Coefficient of variation	Average area-length index	Standard deviation of area-length index	Coefficient of variation
0	1.48	0.39	26	33	9	28
3	4.06	2.13	53	23	8	34
6	1.83	0.61	33	27	8	30
9	2.05	0.55	25	30	9	29
12	1.84	0.55	30	29	8	28
18	1.90	0.74	39	31	9	29
30	2.13	0.79	37	29	7	24
48	2.10	0.71	34	30	9	31
96	1.51	0.61	40	26	10	37

TABLE XIX

DIPHTHEROID BACILLUS

RATE OF GROWTH

Hours of growth	No. of cells per ccm.
0	336,000
3	428,000
6	1,680,000
9	44,480,000
12	122,400,000
15	372,720,000
18	704,320,000
24	787,860,000
30	833,120,000
36	861,520,000
48	858,520,000.

TABLE XX

DIPHTHEROID BACILLUS

STATISTICAL CONSTANTS

Hours of growth	Average length (microns)	Mode (microns)	Median (microns)	Standard deviation (microns)	Coefficient of variation
0	4.80	4.00	4.32	1.66	34.7
1	4.28	4.66	4.33	1.60	32.8
2	5.60	4.66	4.86	2.60	46.4
3	5.80	5.33	5.13	2.14	36.8
4	5.46	4.66	4.86	2.20	40.2
5	4.80	4.66	4.20	2.60	54.2
6	4.86	4.00	4.33	2.06	42.4
7	5.40	5.33	4.86	2.40	44.4
8	5.33	4.66	4.73	3.14	58.8
9	4.73	4.66	4.33	1.06	22.5
10	4.46	4.33	4.06	1.14	25.4
11	3.93	4.00	3.53	1.06	27.1
12	3.33	3.00	3.40	1.06	31.4
13	4.00	4.00	3.60	1.60	40.0
14	3.20	2.66	2.73	1.14	35.4
15	3.80	3.33	3.33	1.14	29.8
16	3.66	3.33	3.20	1.40	39.0
17	4.33	4.00	3.80	1.54	35.5
18	4.06	3.33	3.53	1.34	32.8
21	4.13	4.00	3.73	1.34	32.3
24	3.93	3.33	3.40	1.54	39.0
28	4.73	4.66	4.66	1.66	35.2
32	4.93	4.66	4.40	1.66	33.8
36	4.60	4.00	4.00	1.80	39.7
40	4.80	4.00	4.26	1.66	34.7
44	5.33	4.66	4.73	1.80	33.8
48	5.46	4.00	4.60	2.54	46.3

TABLE XXI

DIPHTHEROID BACILLUS

Hours of growth	Granules per cell	Coeff. of Correlation granules with length
0	1.09	83.3±4.6
3	0.71	78.4±1.8
6	0.16	46.1±3.6
9	0.10	17.3±6.5
12	0.15	42.6±3.9
15	0.21	54.7±4.7
18	0.21	38.4±5.7
24	0.29	64.6±3.9
36	0.42	56.0±3.3
48	0.98	61.8±2.9

TABLE XXII

B. COHAERENS

RATE OF SPORE-FORMATION

Hours of growth	Heavy inoculum Full strength medium	Light inoculum Full strength medium	Heavy inoculum Dilute medium	Light inoculum Dilute medium
2	98% spores	98% spores	98% spores	98% spores
3	92	82	79	72
4	15	13	27	22
5	5	1	19	7
6	1	0	3	2
8	0	0	1	0
11	0	0	4	0
15	1	0	5	0
21	3	1	22	7
28	7	5	42	31
36	20	14	50	43
48	48	46	75	66
72	65	56	92	84

TABLE XXIII

CHOLERA VIBRIO

RATE OF GROWTH

Hour	Cells per cu. mm.
0	2,506
3	2,780
5	3,230
7	21,560
9	194,320
11	725,120
14	2,402,900
18	3,333,200
24	3,465,600
36	4,428,800
48	6,480,800
72	5,964,800
120	5,510,400
168	2,757,200
240	585,000

TABLE XXIV

CHOLERA VIBRIO

AREA-LENGTH INDEX

Hour	Mean	Median	Mode	Standard deviations	Coefficient of variability
0	35.70	28.67	22.5	19.73	55.30
3	40.40	36.58	22.5	20.92	51.78
6	31.45	29.85	27.5	20.15	64.09
9	33.46	31.51	32.5	9.30	27.79
12	28.50	29.20	27.5	9.49	33.31
18	21.20	21.06	22.5	5.99	28.25
24	24.96	22.40	22.5	11.09	44.43
36	24.20	19.25	17.5	16.21	66.98
48	37.70	27.99	17.5	19.74	52.36
72	36.60	31.40	17.5	20.62	56.34
96	34.16	34.88	30.0	21.13	61.86
120	39.20	33.93	27.5	20.49	52.27
168	34.31	35.39	27.5	16.03	46.72
240	34.84	30.48	22.5	18.89	54.23

TABLE XXV

CHOLERA VIBRIO

Hours of growth	Average length of cells	Average index of curvature	Asymmetrical cells
0	2.22μ	5.87	20.5%
3	2.12	3.84	10.5
6	2.57	5.82	7.5
9	2.85	8.22	6.0
12	2.75	8.96	1.0
18	2.86	8.88	2.5
24	2.22	7.82	5.5
36	2.61	5.58	1.5
48	2.78	6.77	26.5
72	2.12	6.46	28.5
96	2.23	6.58	45.5
120	2.03	6.87	31.5
168	1.86	6.46	37.5
240	1.99	4.10	37.0

TABLE XXVI

COLON BACILLUS; RATE OF DEATH

Days of growth	Plate counts Cells per ccm.	Per cent stained cells	Microscopic counts Cells per ccm.
	ALKALINE		
1	2,790,000,000	0.0	2,555,000,000
2	3,650,000,000	3.7	3,820,000,000
3	3,200,000,000	5.1	4,760,000,000
5	2,265,000,000	8.8	5,350,000,000
7	1,700,000,000	11.7	5,500,000,000
10	760,000,000	12.8	5,760,000,000
14	54,000,000	20.1	5,670,000,000
19	8,700,000	23.6	5,600,000,000
25	1,610,000	56.7	5,380,000,000
	NEUTRAL		
1	1,090,000,000	0.0	2,900,000,000
2	2,240,000,000	4.0	4,640,000,000
3	890,000,000	6.5	5,590,000,000
5	322,500,000	11.2	6,050,000,000

TABLE XXVI (continued)

Days of growth	Plate counts Cells per ccm.	Per cent stained cells	Microscopic counts Cells per ccm.
7	226,000,000	27.1	6,190,000,000
10	175,000,000	36.7	5,610,000,000
14	112,200,000	45.2	5,510,000,000
19	106,000,000	57.0	4,600,000,000
25	100,400,000	59.6	3,420,000,000
	SODIUM CHLORIDE		
1	1,540,000,000	1.5	1,320,000,000
2	1,660,000,000	3.1	1,772,000,000
3	1,750,000,000	10.3	1,957,000,000
5	645,000,000	15.8	1,858,000,000
7	230,000,000	21.1	1,611,000,000
10	24,100,000	45.9	1,410,000,000
14	7,600,000	62.8	1,240,000,000
20	1,120,000	82.0	1,075,000,000
	ACID		
1	2,370.000.000	2.2	2,630,000,000
2	2,675,000,000	8.8	3,200,000,000
3	3,400,000,000	13.5	3,026,000,000
5	4,700,000,000	32.6	4,650,000,000
7	1,160,000,000	39.4	5,011,000,000
10	325,000,000	47.8	6,616,000,000
14	85,000,000	58.2	5,420,000,000
19	44,800,000	71.1	5,160,000,000
25	18,200,000	81.8	4,095,000,000
	CALCIUM CHLORIDE		
1	1,140,000,000	12.1	1,330,000,000
2	1,435,000,000	17.0	1,430,000,000
3	1,050,000,000	21.4	1,495,000,000
5	680,000,000	39.6	1,530,000,000
7	600,000,000	43.0	2,270,000,000
10	398,000,000	55.5	2,295,000,000
14	183,000,000	78.0	3,460,000,000
19	100,000,000	84.0	2,600,000,000
25	32,500,000	92.2	2,130,000,000

Table XXVII

Colon Bacillus; Incidence of Various Cell Types in the Death Phase

	Days of Growth									
	1	2	3	5	7	10	14	19	25	Mean
Cell class	ALKALINE									
I	54.5%	51.0%	60.0%	45.0%	61.5%	60.0%	66.5%	53.5%	48.0%	55.55
II	15.5	8.5	8.5	5.0	9.0	7.5	3.0	5.5	8.0	7.83
III	2.0	23.0	20.5	39.5	11.5	15.0	17.5	22.0	29.0	20.00
IV	26.5	9.5	4.0	2.0	11.5	13.0	6.5	4.5	3.5	9.00
V	1.5	1.0	0.5	2.0	2.0	1.0	1.0	4.5	3.5	1.89
VI	0.0	5.0	3.0	2.0	2.0	2.5	2.0	6.5	4.0	3.00
VII	0.0	2.0	4.0	4.5	2.5	1.0	3.5	2.5	3.0	2.55
VIII	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.05
IX	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.05
X	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.11
Index of variation	67.08	67.61	61.80	68.30	63.18	59.31	58.55	86.07	77.45	*67.71*
Cell class	NEUTRAL									
I	42.0	48.5	45.0	57.5	53.5	52.5	45.5	46.0	44.5	48.33
II	11.5	12.5	16.0	11.0	25.0	15.5	10.0	13.0	11.5	14.00
III	40.0	15.5	17.5	19.5	10.5	15.5	18.0	17.5	26.0	20.00
IV	0.5	7.5	4.0	6.0	2.5	5.5	7.5	10.0	5.0	5.39
V	0.5	1.0	3.5	2.0	4.0	0.5	3.5	3.0	4.0	2.47
VI	1.5	11.0	8.0	3.0	1.0	5.5	9.0	5.5	5.5	5.55
VII	3.5	1.0	3.5	1.0	1.0	3.5	3.5	2.5	2.0	2.39
VIII	0.5	0.5	0.0	0.0	2.0	0.5	1.0	2.0	1.5	0.89
IX	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05
X	0.0	2.0	2.5	0.0	0.5	1.0	2.0	0.5	0.0	0.90
Index of variation	64.37	87.82	115.27	55.98	73.17	86.03	119.25	95.41	80.70	*87.56*
Cell class	SODIUM CHLORIDE									
I	62.5	28.5	44.5	46.0	57.0	44.5	60.0	42.0		48.12
II	16.0	30.0	28.5	19.5	25.5	31.5	19.0	37.5		25.93
III	14.5	12.0	10.5	15.0	4.5	8.5	9.0	4.5		9.81
IV	2.5	0.0	1.0	3.0	1.0	3.5	6.0	11.0		3.50
V	2.0	5.0	4.0	7.5	4.0	3.5	4.0	2.5		4.06

TABLE XXVII (continued)

	Days of Growth									
	1	2	3	5	7	10	14	19	25	Mean
VI	1.5	10.0	4.5	3.5	3.0	4.5	1.0	2.0		3.75
VII	0.0	1.0	0.5	2.0	0.5	0.0	0.5	0.0		0.56
VIII	1.0	11.0	4.0	3.0	4.5	3.0	0.5	0.0		3.37
IX	0.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0		0.19
X	0.0	2.0	1.5	0.5	0.0	1.0	0.0	0.5		0.69
Index of variation	50.48	181.55	117.79	96.34	80.24	92.94	55.58	69.18		*93.01*
Cell class	ACID									
I	51.0%	46.0%	36.0%	43.0%	45.0%	47.0%	47.5%	36.0%	35.0%	42.94%
II	21.0	23.0	25.5	20.0	15.0	15.0	13.5	11.5	11.5	17.33
III	5.5	10.5	18.5	12.5	13.5	18.0	11.5	22.5	32.0	16.05
IV	14.0	12.5	11.0	10.0	13.0	8.0	10.5	15.0	3.0	10.77
V	3.0	2.5	1.5	7.0	2.5	5.0	7.5	6.0	3.5	4.50
VI	0.5	4.5	2.5	3.5	4.5	2.5	5.5	2.0	3.0	3.17
VII	3.0	0.0	4.0	2.5	4.0	2.0	2.5	5.5	12.0	3.94
VIII	2.0	0.5	0.5	1.0	1.0	1.0	1.0	0.5	0.0	0.83
IX	0.0	0.5	0.0	0.5	1.0	0.5	0.0	0.5	0.0	0.33
X	0.0	0.0	0.5	0.0	0.5	1.0	0.5	0.5	0.0	0.33
Index of variation	82.73	75.10	88.72	91.12	106.37	95.69	95.81	109.76	85.73	*93.42*
Cell class	CALCIUM CHLORIDE									
I	48.0	38.0	42.0	41.5	42.0	32.0	39.0	35.0	42.0	39.94
II	23.0	23.5	23.5	21.0	25.0	8.0	17.5	10.0	10.0	17.94
III	7.0	6.5	5.0	11.0	8.0	24.5	8.0	24.5	24.0	13.17
IV	1.0	5.0	7.0	1.5	1.5	2.0	4.0	3.0	2.0	3.00
V	10.5	14.5	9.5	13.5	11.5	11.0	9.0	8.5	6.5	10.50
VI	7.5	4.5	9.0	6.5	3.0	5.0	6.0	4.5	5.5	5.72
VII	2.5	5.0	0.5	1.5	1.0	3.5	3.0	3.5	2.0	2.50
VIII	0.0	2.0	0.5	1.5	1.0	4.5	4.0	2.5	3.5	2.17
IX	0.0	0.5	0.5	1.0	5.5	8.5	8.0	6.5	3.5	3.78
X	0.5	0.5	2.5	1.0	1.5	1.0	1.5	2.0	1.0	1.29
Index of variation	88.06	123.78	127.97	118.30	161.35	226.02	220.21	200.83	148.63	*157.25*

APPENDIX

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